DOCKET NO.:ISIS-5213 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Phillip Dan Cook and Brett P. Monia

Serial No.: **10/601,242** Group Art Unit: **1633**

Filing Date: **June 20, 2003** Examiner: **Janet L. Epps Ford**

For: **Gapped 2'-Modified Oligonucleotides**

DECLARATION OF C. FRANK BENNETT UNDER 37 C.F.R. § 1.132

I, C. Frank Bennett, hereby declare that:

1) I currently am employed as Vice President of Antisense Research at Isis Pharmaceuticals. I have held this position since 1995. I have been involved in the development of antisense oligonucleotides as therapeutic agents, including research on the application of oligonucleotides for inflammatory and cancer targets, oligonucleotide delivery and pharmacokinetics since at least 1989. I have published more than 90 papers in the field of antisense research and development. I received my B.S. degree in Pharmacy from the University of New Mexico, Albuquerque, New Mexico and my Ph.D. in Pharmacology from Baylor College of Medicine, Houston, Texas.

2) I am aware of published articles that describe a correlation between the affinities of antisense oligonucleotides for their targets and the structures of the oligonucleotides. For example, in "The ups and downs of nucleic acid duplex stability: structure-stability studies on

chemically-modified DNA;RNA duplexes," Freier et. al., *Nucleic Acid Research*, 1997, Vol. 25, No. 22, 4429-4443 (copy attached as Exhibit 1) studies that correlate the structures of antisense oligonucleotides having more than 200 modifications to their binding affinities for their targets are reviewed.

3) I am aware of published articles that describe nuclease resistance and antisense activity of oligonucleotides. For example, in "Nuclease Resistance and Antisense Activity of Modified Oligonucleotides Targeted to Ha-ras," Monia et. al., *J. Biol. Chem.*, Vol. 271, No. 24, 1996, 14533-14540 (copy attached as Exhibit 2), nuclease stability and antisense activity of various phosphorothioate and phosphodiester oligonucleotides are described.

4) I am aware that DNA includes nucleotide units having 2'-deoxy-*erythro*-pentofuranosyl sugar moties, *i.e.*, 2'-deoxyribose moties. I am further aware of published articles that describe the properties of RNase H enzymes that hydrolyze RNA in RNA-DNA duplexes in which at least some of the nucleotide subunits of the DNA portion of the duplexes have 2'-deoxy-*erythro*-pentofuranosyl sugar moties. For example, in "Properties of Cloned and Expressed Human RNase H1," Wu et. al., *J. Biol. Chem.* , Vol. 274, No. 40, 1999, 28270-28278 (copy attached as Exhibit 3), properties of RNase H enzymes including human RNase H1 enzymes are described including positional preference cleavage of the RNA target in an RNA-DNA duplex.

5) I am aware of a number of published articles and books that describe the unique and beneficial properties of oligonucleotides, including published reports summarizing the antisense activity of oligonucleotides *in vivo* against a variety of targets under a variety of

conditions. For example, "Basic Principles of Antisense Therapeutics" in *Antisense Research and Application*, Stanley T. Crooke, ed., chapter 1, 1-50, 2001 ("the Crooke chapter"; copy attached as Exhibit 4) reports that antisense oligonucleotides exhibited *in vivo* activity against 49 targets when administered to five species of animals using 11 modes of administration (*see, e.g.,* Table 1).

6) I have reviewed the claims that I understand to currently be pending in the above-identified patent application. The claims refer to oligonucleotides having the following three features: (1) at least one of the nucleotide units of the oligonucleotide is functionalized to increase the nuclease resistance of the oligonucleotide; (2) at least one of the nucleotide units bears a substituent group that increases the binding affinity of the oligonucleotide for its target nucleic acid; and (3) a plurality of the nucleotide units have 2'-deoxy-*erythro*-pentofuranosyl sugar moieties that are consecutively located within the oligonucleotide.

7) In "Antisense Oligonucleotide-Based Therapeutics" in *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*, Nancy Smyth Templeton, ed., 2003, chapter. 19, 347-374 (copy attached as Exhibit 5), I and my co-authors reported on clinical testing involving six oligonucleotides possessing these features. Each of the oligonucleotides had a different molecular target and was used to treat cancer, rheumatoid arthritis, diabetes, or multiple sclerosis.

8) I believe that those skilled in the art following the teachings provided in this patent application could readily prepare oligonucleotides possessing the above-noted three features with an expectation that the oligonucleotides would exhibit antisense activity *in vivo* against a

target of interest. In my view, the patent application provides sufficient disclosure for those skilled in the art to practice the presently claimed methods without undue experimentation.

9) I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

11-16-05 C. Frank Bennett

Date C. Frank Bennett

Nucleic Acids Research, 1997, Vol. 25, No. 22 4429–4443

The ups and downs of nucleic acid duplex stability: structure–stability studies on chemically-modified DNA:RNA duplexes

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ABSTRACT

In an effort to discover novel oligonucleotide modifications for antisense therapeutics, we have prepared oligodeoxyribonucleotides containing more than 200 different modifications and measured their affinities for complementary RNA. These include modifications to the heterocyclic bases, the deoxy-ribose sugar and the phosphodiester linkage. From these results, we have been able to determine structure–activity relationships that correlate hybridization affinity with changes in oligonucleotide structure. Data for oligonucleotides containing modified pyrimidine nucleotides are presented. In general, modifications that resulted in the most stable duplexes contained a heteroatom at the 2′-position of the sugar. Other sugar modifications usually led to diminished hybrid stability. Most backbone modifications that led to improved hybridization restricted backbone mobility and resulted in an A-type sugar pucker for the residue 5′ to the modified internucleotide linkage. Among the heterocycles, C-5-substituted pyrimidines stood out as substantially increasing duplex stability.

INTRODUCTION

The high affinity and specificity of Watson–Crick hybridization has made oligonucleotides attractive agents for diagnostic and therapeutic applications. Although unmodified DNA oligonucleotides have been reported to demonstrate antisense activity in cell assays, much research has been devoted to the discovery of modified oligonucleotides as antisense therapeutics (1). The primary goal of these modifications has been to improve biostability and cellular uptake of the oligonucleotides and to optimize tissue and cell distribution for a particular molecular target. It is important, however, that modified oligonucleotides maintain the hybridization characteristics of unmodified DNA. The mechanism of action of antisense oligonucleotides requires specific hybridization of the oligonucleotide at its complementary site on the mRNA. The importance of hybridization is demonstrated by the correlation of antisense activity observed in cell assays (2–5) and *in vivo* (6) with hybridization affinity and T_M. Described below is a strategy using six test sequences for evaluation of hybridization properties of chemically-modified oligonucleotides to RNA complement. Over 200 modifications were tested as part of our antisense drug discovery effort. The behavior of these modifications in this screening system will be discussed.

METHODS

Strategy for evaluation of oligonucleotide modifications

To maximize the number of oligonucleotide modifications that can be prepared and evaluated for utility in antisense applications, we adopted a two-phase strategy. In the first phase, only the modified T nucleoside was prepared as a 5′-*O*-DMT-protected phosphoramidite and a series of oligonucleotides containing only modified thymidines was prepared. Alternatively, if the modification was in the phosphodiester backbone, a T-T dimer containing the modified backbone between two thymidine residues was prepared. Block coupling of these modified dimers resulted in oligonucleotides with backbone modifications between consecutive T residues. Hybridization and nuclease resistance properties for this series of oligonucleotides with modifications only at the T residues or T-T linkages were evaluated *in vitro*. Only if the hybridization affinity, hybridization specificity and nuclease resistance of these modified oligonucleotides met some minimum requirements, modified amidites were prepared for the other nucleobases and the modification was incorporated into antisense oligonucleotides for testing in a cellular assay. This strategy has proved effective. Usually, synthesis of the modified T nucleoside phosphoramidite required fewer steps than the corresponding A, C or G amidites so the initial evaluation could be made rapidly. In general, hybridization properties of oligonucleotides which contain modifications only on a single nucleobase have been predictive of properties for uniformly modified oligonucleotides or 'gapmers' which contain a stretch of DNA flanked by regions of modification (3,7,8). More important, modifications that bind weakly to complementary RNA in this series have not demonstrated good antisense activity (K. H. Altmann, B. Monia and N. Dean, unpublished results). Thus preliminary evaluation of hybridization using only modified thymidines has been predictive of the value of a modification for antisense applications.

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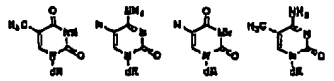
Table 1. Sequences containing modified thymidine used for hybridization studies

sequence number	modified sequence[1]	# of modifications: T nucleosides	# of modifications: T–T dimers
seq1	TCCAGGTTCCTCGCAAC	4	NA
seq2	CTCGTACGTTTCCGTGTCC or CTCGTACG-t-tCCGGTCC	1	1
seq3	CTCGTACttCCGGTCC or CTCGTACt-t-tCCGGTCC	4	2
seq4	GCCtttttttGCG or GCGt-t-t-t-t-t-tGCG	10	5
seq5	TTTTtCTCTCTCTCT or TTTt-tCTCTCTCTCT	1	1
seq6[2]	tttttttttttT or t-t-t-t-t-t-t-t-t-t-t-T	14	7

[1]t represents a modified thymidine nucleoside. t-t represents a TT dimer in which the phosphodiester linkage between the thymidines has been modified.
[2]This sequence contains modified thymidine and deoxycytidine residues.

Table 2. Effect of 5-methyl pyrimidine substitution on T_M



T dC dU 5-methyl dC

mod #		ΔT_M per mod (parent is DNA): *seq1*	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*
	PARENT FOR ALL MODIFICATIONS						
	T(dC)	0.0 (62.3)[1]	0.0 (60.3)[1]	0.0 (61.8)[1]	0.0 (50.2)[1]	0.0 (52.7)[1]	0.0 (52.7)[1]
	1ST GENERATION MODIFICATIONS						
(1)	U(dC)	-0.6	-1.2	-0.2	-0.4	-0.1	-0.3
(2)	U(5-MeC)						+0.0
(3)	T(5-MeC)						+1.1

[1]Values in parentheses are the T_M (°C) for the unmodified DNA with its RNA complement.

Hybridization was evaluated using absorbance versus temperature profiles. The technique required only 2–4 OD units of modified oligonucleotide and reproducible results were easily obtained. Although T_M can be precisely measured, it is not a thermodynamic parameter and does not directly measure hybridization affinity. $\Delta G°_{37}$ is the appropriate parameter for evaluating hybridization affinity. It represents the free energy difference between duplex and single strands at 37°C. Unfortunately, for oligonucleotides longer than about 10 residues and ionic strengths less than –0.5 M, coil to helix transitions tend to be non-two state (9–12) and this was observed for many of our transitions. $\Delta G°_{37}$ obtained from the melting curves was sensitive to analysis method, particularly to how baselines were drawn (13–15). T_M, on the other hand, was much less sensitive to analysis method. Consideration of the thermodynamic equations demonstrates that for changes in T_M <25°C and changes in $\Delta H°$ <25%, ΔT_M correlates quite well with $\Delta\Delta G°_{37}$. This correlation has also been observed experimentally (7,8). Thus T_M and ΔT_M were used to evaluate the effect of chemical modification on duplex stability. [Note: throughout this manuscript, we use the phrases 'duplex stability' and 'hybridization affinity' to refer to $\Delta G°_{37}$, the free energy difference between duplex and single strands at 37°C.]

The six sequences used for the initial hybridization studies are listed in Table 1. They included sequences with single modifications interspersed between unmodified residues (*seq1*, *seq2* and *seq5*), sequences with short or long continuous stretches of modified residues (*seq3* and *seq4*, respectively) and one sequence (*seq6*) that was fully modified, except for the 3′ terminal nucleoside. Our primary interest was in the utilization of these modifications for antisense applications so we focused on hybridization of the modified oligonucleotides to complementary RNA.

To test the effect of our modifications in a uniformly modified oligonucleotide, the modified C amidite was synthesized and *seq6* was prepared. We opted to prepare the C amidite rather than to test homo-T oligomers for hybridization. When mixed with oligo-A, oligo-T can form many complexes including triple-stranded structures and high molecular weight aggregates with staggered duplexes (16). Due to the symmetry of the sequence, both parallel and antiparallel hybridization is possible. These complex structures can be difficult to characterize due to slow hybridization and coexistence of multiple species. Results with such complex structures can also be misleading. For example, triple-stranded complexes formed by PNA T_{10} and dA_{10} led to the conclusion that T_M values for short PNA–DNA duplexes were 50°C higher than their DNA–DNA counterparts (17,18). However, later work with mixed sequences demonstrated that, at physiological ionic strength, the ΔT_M value for a PNA–DNA 10mer duplex was only 17°C (19).

T_M measurements

Absorbance versus temperature curves were measured as described previously (7). Each sample contained 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, 4 μM modified oligonucleotide and 4 μM complementary, length matched RNA. ΔT_M per modification was calculated by subtracting T_M of the unmodified DNA–RNA parent duplex and dividing by the number of modified residues in the sequence. Average ΔT_M per substitution was calculated by summing the ΔT_M values for all oligonucleotides containing that modification and dividing by the total number of substitutions. Averages calculated by this method weigh each oligonucleotide by the number of substitutions it contains.

RESULTS

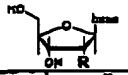
T_M values for the unmodified controls

T_M values for the six unmodified DNA sequences versus their RNA complements are listed in Table 2. In addition, ΔT_M values are listed for the same sequences containing dU (1) (bold numbers in parentheses refer to modification numbers from the tables and figures) and for *seq6* with 5-methyl dC (2–3). Each substitution of dU for T resulted in an average change of about –0.5°C in T_M and substitution of 5-methyl dC for dC resulted in an average increase of about +0.5°C per substitution (20). For all modifications discussed below, ΔT_M values were calculated relative to the unmodified DNA parent (containing T and dC) even though some of the sugar and backbone modifications were prepared on dU rather than T or on 5-methyl C rather than dC. For each modification, the nucleobase is listed. If dU was used, a destabilization of approximately –0.5°C per substitution would be expected in addition to any stabilization or destabilization caused by the modified sugar or backbone.

Sugar modifications

Effect of 2′-sugar substitution. Table 3 lists ΔT_M values for oligonucleotides containing substitutions at the 2′ position of deoxyribose. It is clear from Table 3 that the exact value of ΔT_M per substitution depended on sequence. Thus, effects of modifications on duplex stability can be compared quantitatively only if the same sequences were used for all modifications. Trends,

Table 3. Effect of 2′ sugar substitution of T_M



#	Modification: hetero-cycle	Modification: 2′-substituent	ΔT_M per mod (parent is DNA): seq1	seq2	seq3	seq4	seq5	seq6	reference
(4)	U(dC)	-F	+0.6	+0.1			+1.0	+1.3	21
(5)	T(5-dC)	-F					+1.7	+2.5	P. Martin, unpublished results
(6)	U(dC)	-OH						+0.4	D. Hüsken, unpublished results
(7)	U(dC)	-O-CH_3	+0.2	+1.2	+0.5	+0.3	+0.5	+0.8	92, 93, 94
(8)	T(5-dC)	-O-CH_3	+1.1		+0.8	+1.1	+1.2	+1.4	30
(9)	U(dC)	-O-C_2H_5-CH_3					+0.6	+0.7	7, P. Martin, unpublished results
(10)	T(5-dC)	-O-C_2H_5-CH_3	+0.7	+1.1	+0.7	+0.8	+1.4	+1.4	P. Martin, unpublished results
(11)	T	-O-CH-$(CH_3)_2$				-0.4	-0.1		30
(12)	T	-O-CH_2-CH=CH_2	+0.8		+0.4	+0.8	+1.3		94, 95, 96, P. Martin, unpublished results
(13)	T	-O-C_2H_4-CH_3	+0.6		+0.6	+0.8	+1.2		7, P. von Matt, unpublished results
(14)	U	-O-C_2H_4-CH_3					-0.1		7
(15)	T	-O-C_3H_7	-0.2		-1.1	NC[1]	-1.6		A. Waldner, unpublished results
(16)	T	-S-C_2H_5	-3.6		-3.8	NC[1]	-6.4		P. Martin, unpublished results
(17)	T(dC)	-CH_3	-1.3	-1.1	-2.3	NC[1]	-2.2	-2.5	24
(18)	T	-CH_2F					-3.1		24
(19)	T	-CF_3					-4.7		24
(20)	U	-CH_2-CH_3	-3.1	-5.8	-3.5	NC[1]	-4.4		97
(21)	U	-CH_2-CH=CH_2		-5.1		NC[1]			97
(22)	T	-CH_2-CH=CH_2	-2.4	-5.0	-3.7	NC[1]	-4.7		97
(23)	T	-C_4H_9	-3.6	-6.4	-3.7	NC[1]			24
(24)	U	-CH=CH-C_2H_5	-3.9	-7.3	-3.4	NC[1]			97
(25)	T	-CH_2-OH		-2.9	-3.1	NC[1]	-2.6		24
(26)	T	-CH_2-O-CH_3	-2.1	-4.3	-1.2	NC[1]	-3.4		24
(27)	T	-CH_2-CH_2-CH_2-O-CH_3	-2.0		-3.2	NC[1]	-5.9		K.-H. Altmann, unpublished results
(28)	U	-O-CO-NH-(CH_3)	-2.3	-3.2	-5.8	NC[1]			M. Manoharan, manuscript in preparation
(29)	U	-O-CO-NH-CH_2-CH_2-N-$(CH_3)_2$		-5.7					M. Manoharan, manuscript in preparation
(30)	U	-O-C_2H_4-NH_2	+0.2	-2.6	-0.5	-0.6			27,98
(31)	T	-O-C_2H_4-NH_2	+1.3				-1.9		P. Martin and K. H. Altmann, unpublished results
(32)	U	-O-CH_2-$C_{15}H_9O_2$[2]					+2.1	+1.6[3]	P. Martin, unpublished results
(33)	U(dC)	-O-CH_2-O-CH_2-CH_3						+0.0	P. Martin, unpublished results
(34)	T	-O-CH_2-O-CH_2-Cl		-0.9	-0.2	0.0	+1.2		P. Martin, unpublished results
(35)	T(dC)	-O-C_2H_4-O-CH_3	+0.9	+1.6	+0.9	+1.2	+1.2	+1.7	30,32
(36)	T	-O-C_3H_6-O-CH_3					+0.4		P. Martin, unpublished results
(37)	T	-$(O$-$C_2H_4)_2$-O-CH_3					+0.9		30
(38)	T(5-dC)	-$(O$-$C_2H_4)_3$-O-CH_3	+1.2	+0.7	+0.8	+1.1	+1.1	+1.7	30
(39)	T	-$(O$-$C_2H_4)_4$-O-CH_3				+0.5	+0.4		30
(40)	T	-$(O$-$C_2H_4)_3$-O-C_2H_4-CH_3					+0.2		P. Martin, unpublished results
(41)	T	-O-C_2H_4-CF_3				+1.1	+1.0		P. Martin, unpublished results
(42)	T	-O-C_2H_4-OH					+1.3		P. Martin, unpublished results
(43)	T	-O-C_2H_4-F	-1.4	+0.3	+1.1	+1.3	+1.7		P. Martin, unpublished results
(44)	T	-O-CH_2-$CH(CH_3)$-F				+1.3	+0.2		P. Martin, unpublished results
(45)	T	O-CH_2-$CH(CH_2OH)$-OH				+1.2	+1.5		P. Martin, unpublished results
(46)	T	O-CH_2-$CH(CH_2OH)$-OH	+1.2	+0.8	+0.9		+1.5		30
(47)	T	-O-CH_2-$CH(CH_2$-$OCH_3)$-OCH_3					+1.1		P. Martin, unpublished results
(48)	T	-O-CH_2-$CH(CH_3)$-OCH_3					+1.0		P. Martin, unpublished results
(49)	T	-O-CH_2-$CH(CH_2$-O-$C_{15}H_{30}$-$CH_3)$-OCH_3					-0.3		P. Martin, unpublished results
(50)	T	β-CH_3	-2.3	-3.9	-3.1	NC[1]	-3.3		24
(51)	T	α-CH_3	-1.9	-5.2	-3.4	NC[1]	-3.5		24

[1]NC, non-cooperative transition.
[2]The structure of this anthraquinone derivative is given in Figure 1C.
[3]This oligonucleotide contained substitutions at positions 4 and 13 only.

however, were consistent across all sequences studied. Modifications that stabilized the duplex did so for all sequences; modifications that destabilized the duplex reduced T_M for all sequences.

Figure 1 plots the average ΔT_M per substitution for the substitutions in Table 3. Among the 2′ substitutions reported here, a 2′-fluoro substituent (4–5) was the most stabilizing. In general, 2′-*O*-alkyl substitution (7–14) also stabilized the duplex, with smaller substituents resulting in greater duplex stability than larger ones. A clear correlation between substituent size and duplex stability has been reported previously for a large series of 2′-*O*-alkyl substitutions (7) and is confirmed by the data in Figure 1A. The improved hybridization of 2′-F and 2′-*O*-R-substituted oligonucleotides to complementary RNA has been attributed to the tendency of these electronegative substituents to shift the conformational equilibrium in the sugar moiety toward the northern (C3′-*endo*) conformation consistent with the A-form geometry of RNA duplexes (7,21–23). Destabilization by larger 2′-*O*-alkyl substitutions, on the other hand, may be caused by steric interference of the larger alkyl chains with other parts of the duplex or disruption of water structure in the minor groove (7).

In contrast to the increase in duplex stability observed with electronegative substituents at the 2′ position, 2′-sulfur linked (16) or 2′-carbon linked (17–27) modifications were very destabilizing (Fig. 1B). Destabilization due to 2′-α alkyl substitution was explained by the tendency of these substituents to shift the conformational equilibrium of the sugar toward the C2′ *endo* pucker and away from the C3′ *endo* pucker found in RNA duplexes (24). Destabilization by 2′-*S*-phenyl (16), 2′-*S*-methyl (25) and 2′-amino (26) substitution likely has a similar explanation.

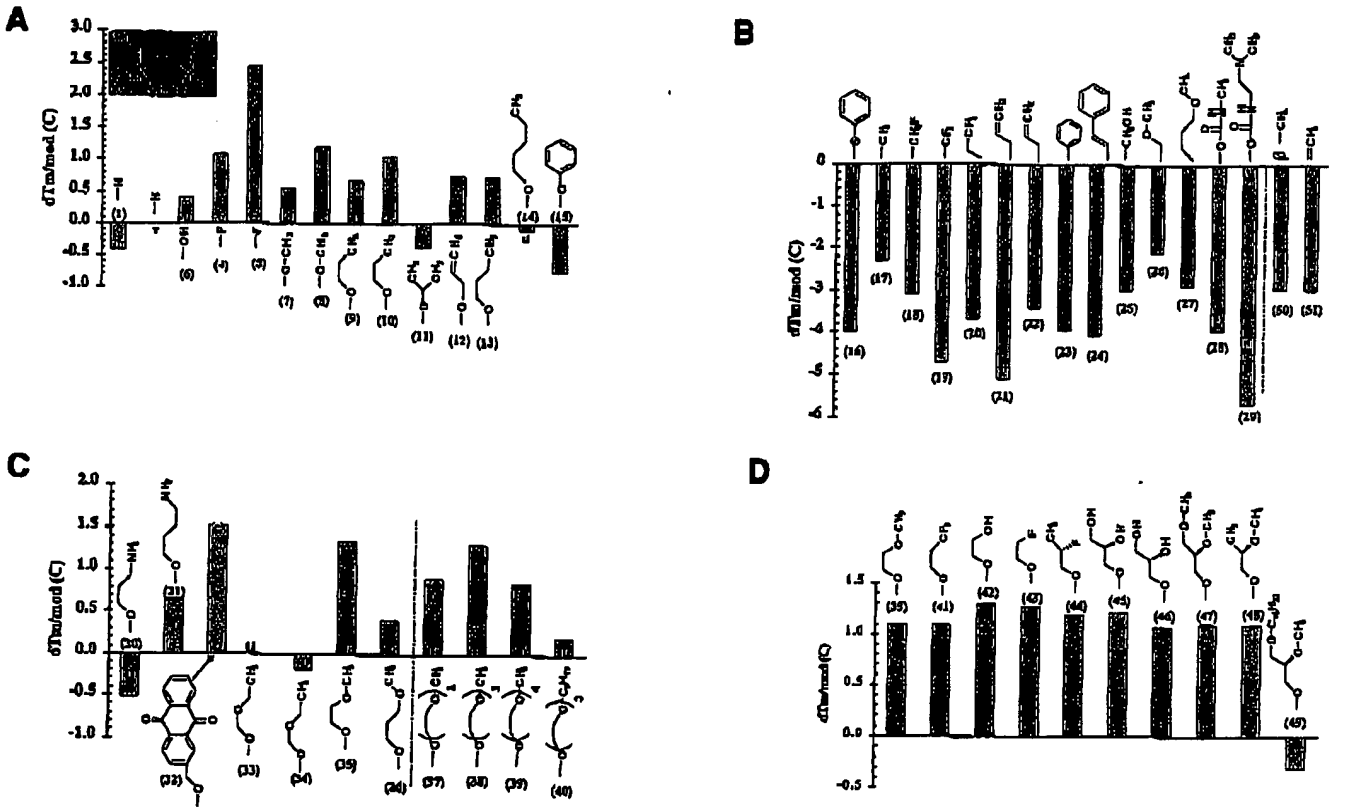


Figure 1. Average ΔT_M (°C) per substitution for 2′-substituted oligonucleotides. (A) 2′-fluoro-, 2′-*O*-alkyl-, 2′-*O*-allyl- and 2′-*O*-phenyl-substituted oligonucleotides; (B) oligonucleotides with 2′-sulphur or 2′-carbon linked substitutions or 2′-carbamate linkages; (C) 2′-*O* substitutions with heteroatoms in the side chain; (D) substitutions with the structure 2′-O-CH_2-CH_2-X where X = O, F or CF_3. For Figures 1–7, bold numbers in parentheses refer to the modification numbers in Tables 3–15.

In contrast, 2′-β-methyl substitution (**50**) drives the sugar equilibrium toward C3′ *endo* but the 2′-β-methyl substituent causes steric conflict in an A-form duplex (24). Also shown in Figure 1B are two 2′-carbamate substitutions (**28–29**). These substitutions were very destabilizing. Apparently the rigid carbamate causes steric interference in the minor groove (M. Manoharan, manuscript in preparation).

Figure 1C reports the effect of additional heteroatoms in 2′-*O*-R substituents. 2′-*O*-amino-alkyl substitution (**30–31**) results in a zwitterionic oligonucleotide and, on average, had little effect on duplex stability. Similar results have been reported for 2′-*O*-amino-propyl substitution in other sequences (27). The 2′-*O*-anthraquinolylmethyl U modification (**32**) was very stabilizing suggesting the anthraquinone may intercalate into the hybrid duplex. Similar stabilization has been observed for oligonucleotides with intercalators attached to the 3′ end (28,29). When a second oxygen was incorporated into the 2′-*O*-alkyl side chain, the 2′-*O*-methoxy-ethyl modification (**35**) stood out as uniquely stabilizing. This stabilization is apparently associated with the ethylene glycol motif; 2′ substituents with as many as four ethylene glycol units (**37–39**) still stabilized the duplex. Even a nonyl group was well tolerated at the end of the ethylene glycol chain (**40**). This contrasts with a destabilization of 2–3 °C per substitution reported for 2′-*O*-nonyl substitution (7). The observation that 2′-O-$(CH_2)_n$-O-CH_3 substitution stabilized the duplex for $n = 2$ (**35**) but had little effect on duplex stability for $n = 1$ (**34**) or $n = 3$ (**36**) led to the hypothesis that, due to the *gauche* effect, the second oxygen of the 2′-ethylene glycol results in a conformation of the side chain consistent with duplex formation (30–32). Results in Figure 1D provide further support for this hypothesis. Substituents with the structure 2′-O-CH_2-CHR-X where X = OH, F, CF_3 or OCH_3 and R = H, CH_3, CH_2OH or CH_2OCH_3 (**41–48**) all resulted in substantial stabilization of the duplex. This suggests an electronegative group at X and any group at R results in duplex stabilization. The only exception was substitution with a very long hydrocarbon on the second carbon (R = $OC_{16}H_{33}$) (**49**) which was destabilizing.

Table 4. Effect of 3′-substituted thymidines on T_M

Modification			ΔT_M per mod (parent is DNA)						
#	R_1	R_2	seq3	seq3	seq3	seq4	seq5	seq6	ref.
(52)	$-CH_3$	-H	-0.1	-1.3			-1.5		24
(53)	$-CH_3$	$-O-CH_3$	-1.4	-4.6			-1.5		24

Effect of 3′-β substitution. Table 4 reports ΔT_M values for oligonucleotides modified at the 3′ position. 3′-β methyl substitution (**52**) resulted in reduced duplex stability. Additional 2′-α-*O*-methyl substitution (**53**) decreased duplex stability even further. Destabilization by these substitutions has been attributed to a strong preference of the 3′-β methyl nucleoside for the 2′ *endo* conformation which is incompatible with an A-form duplex and to unfavorable steric interactions in the modified duplex (24).

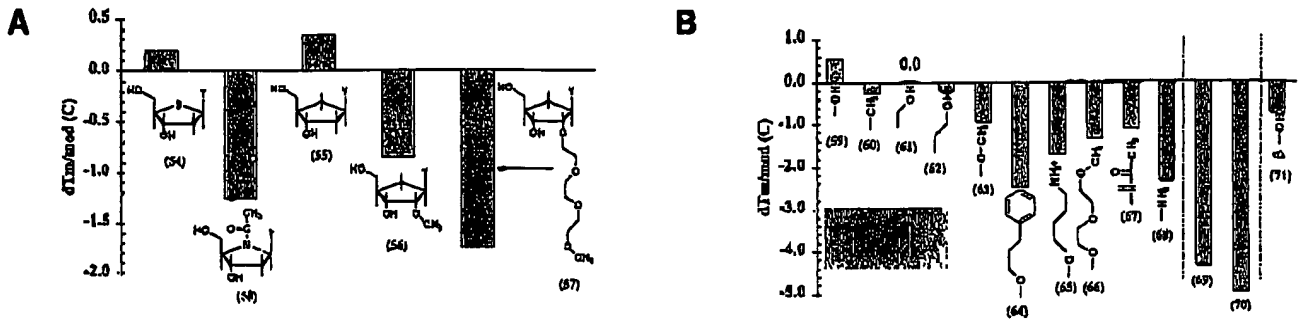


Figure 2. Average ΔT_M (°C) per substitution for oligonucleotides containing replacements for the furanose oxygen O4'. (A) Replacement of the ring oxygen with S, CH_2 or $NCOCH_3$ and (B) substitution at the 6' carbon of carbocyclic nucleosides.

Table 5. Effect of 4' oxygen substitution on T_M

#	Modification -X-	-R	ΔT_M per mod (parent is DNA) seq1	seq2	seq3	seq4	seq5	seq6	ref.
(54)	-S-	-H				+0.2			34
(55)	$-CH_2-$	-H		+0.5		+0.4			99
(56)	$-CH_2-$	$-O-CH_3$		-1.9	-0.7	+1.0	+1.1		37
(57)	$-CH_2-$	$-(O-C_2H_4-)_2-O-CH_3$	-1.1	-2.9	-2.6	NC[1]	+0.2		37
(58)	$-N(COCH_3)-$	-H	-1.2	-0.6	-1.5				36

[1]NC, non-cooperative transition.

Table 6. Effect of 1' and 6' substituted carbocyclic nucleoside analogs on T_M

#	Modification -R1	-R2	ΔT_M per mod (parent is DNA) seq1	seq2	seq3	seq4	seq5	seq6	reference
(59)	-OH	-H	+0.4		+0.2	+0.8	-0.8	+0.6	37
(60)	$-CH_3$	-H	-1.5	-0.8	-0.5	-0.2	-0.1	+0.1	100
(61)	$-CH_2OH$	-H	-1.0			+0.2	-0.1	+0.2	100
(62)	$-C_2H_4OH$	-H					+0.2		K.-H. Altmann, unpublished results
(63)	$-O-CH_3$	-H	-1.3	+0.2	-0.7	-0.9	-2.0		37
(64)	$-O-C_2H_4-C_2H_5$	-H	-2.8	+2.8	+1.7	NC	-4.0		37
(65)	$-O-C_2H_4-NH_2$	-H	-1.4	-1.8		+1.9	-1.1		37
(66)	$-(O-C_2H_4-)_2-O-CH_3$	-H					-1.8	-1.5[1]	37
(67)	$-NH-CO-CH_3$	-H					-1.1		K.-H. Altmann, unpublished results
(68)	$-NH_2$	-H	-3.0	-4.0	-1.6		-1.1		K.-H. Altmann, unpublished results
(69)	-H	$-CH_3$	-5.2	-5.9	-3.3	NC[1]	-3.7		38
(70)	$-CH_3$	$-CH_3$	-6.1	-7.5	-3.8	NC[1]	-2.8		K.-H. Altmann, unpublished results
(71)	β-OH	-H				-0.4	-4.3		K.-H. Altmann, unpublished results

[1]NC, non-cooperative transition.
[2]The heterocycles for this oligonucleotide were thymine and cytosine.

Effect of 4'-oxygen replacement. Table 5 lists ΔT_M values for oligonucleotides in which the ring oxygen of the furanose has been replaced with sulfur (54), carbon (55) or nitrogen (58). Averaged values are plotted in Figure 2A. Replacement of the oxygen with S (54) or CH_2 (55) had little effect on T_M consistent with the DNA-like conformation adopted by these nucleosides (33–35). 2'-*O*-methyl (56) or 2'-*O*-methoxy-diethoxy-ethyl (57) substitution on the carbocycle destabilized the duplex. This contrasts with the stabilizing effect these 2' substituents had on a ribonucleoside and emphasizes the importance of the *gauche* effect between the ring oxygen and the 2' oxygen in duplex stability.

In contrast to the slightly stabilizing effect of S or CH_2, replacement of the ring oxygen with an *N*-acetyl moiety (58) destabilized the duplex. It has been suggested that this destabilization is due to distortion in duplex structure caused by the acetyl group or the tertiary amide bond (36). ΔT_M values for oligonucleotides containing carbocyclic nucleosides modified at the 6' position (in the carbocyclic nucleoside, the CH_2 which replaces the ring oxygen is designated 6') (59–71) are reported in Table 6 with average values plotted in Figure 2B. 6'-α substitution with a methyl (60), hydroxymethyl (61), hydroxyethyl (62) or a hydroxy (59) group was well tolerated while 6'-α-OR (63–66), 6'-α-amino (68) or 6'-α-acetylamino (67) substitution was destabilizing. It has been suggested that the stability of hybrid duplexes containing 6'-α-OH substitution is due to favorable solvation of the hydroxyl modified duplexes and their potential for H-bonding with adjacent residues (37). Model building suggested that 6'-α substituents can be accommodated in an A-form duplex so destabilization by the 6'-α-OR and other substituents may be due to unfavorable solvation effects.

Oligonucleotides containing 1'-β methyl-substituted carbocyclic nucleosides (69–70) hybridized very poorly (Table 6 and Fig. 2B). This destabilization might be due to a tendency of the 1'-α methyl carbocyclic nucleoside to adopt a 1' *exo* conformation which is inconsistent with an A-form duplex structure (38). In contrast to 6'-α-hydroxy substituents, 6'-β-OH groups (71) led to duplex destabilization. This might be related to unfavorable effects on base conformation such as a preference of the base for a *syn* rather than the usual *anti* orientation.

Effect of bicyclic sugars. In an attempt to pre-organize the antisense oligonucleotide into a structure compatible with A-type duplex formation, several bicyclic sugar modifications have been investigated. Structures for four of such conformationally constrained building blocks and averaged values for ΔT_M per substitution are shown in Figure 3 and exact T_M data for our sequences are listed in Table 7. Among these bicycles, only the 4'-6'-methano carbocyclic thymidine (73) stabilized the duplex. DNA:RNA duplex stabilization correlates with the tendency of this nucleoside to adopt a northern conformation (39,40). The 1'-6'-methano carbocyclic thymidine (72), in contrast, favors the Southern conformation and resulted in a decrease in duplex stability (41). The other two bridged nucleosides (74–75) destabilized the duplex substantially. Destabilization by (75) may be due to the rigidity of this modification.

Table 7. Effect of bicyclic modification or cyclobutyl substitution on T_M

Modification #[1]	ΔT_M per mod (parent is DNA)						reference
	seq1	seq3	seq4	seq5	seq6		
(72)	-1.8	-0.9	+1.1	-0.8	-1.8	-1.1	41
(73)					+0.8	-2.1[2]	39,40
(74)		-2.3					H. Moser and R. Mah, unpublished results
(75)	-4.5	-5.8	-3.9	NC[3]	-5.1		101
(76)	-3.3	-3.7	-3.4	NC[3]			G. Buschang and F. Gasparini, unpublished results
(77)	-1.3	-4.0	-3.2	NC[3]			G. Buschang and F. Gasparini, unpublished results

[1]Structures for these modifications are given in Figure 3.
[2]This oligonucleotide had a single modification in position 10.
[3]NC, non-cooperative transition.

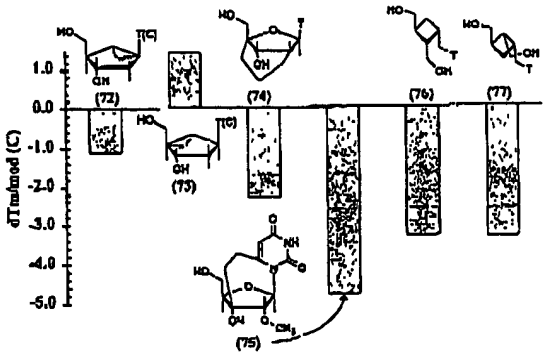


Figure 3. Average ΔT_M (°C) per substitution for oligonucleotides containing bicyclic sugar analogs.

Furanose replacement by four-membered rings. Two cyclobutyl nucleoside analogs (**76–77**) were incorporated into oligonucleotides. They greatly destabilized the duplex (Fig. 3).

Nucleobase modifications

Effect of substitution at the 5 or 6 position of thymine. ΔT_M values for oligonucleotides containing substitutions at the 5 and 6 positions of uracil are reported in Table 8 with average values plotted in Figure 4A. Removal of the 5-methyl group of T to generate dU (**1**) resulted in a slight decrease of duplex stability. Substitution of the 5-methyl group with a halogen (**78–80**) had little effect and substitution with a methoxy-ethoxy-methyl group (**83**) was destabilizing. Among the bases substituted at the 5 position, 5-propynyl dU (**81**) stood out as most stabilizing. This stabilization has been explained by increased stacking (**42**) and has also been observed for 5-methylthiazole-substituted dU (**43**) and tricyclic dC analogs (**44**).

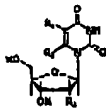
A single positively charged amino-propyl group at the 5 position of U (**82**) had a slight positive effect on duplex stability at this ionic strength. Slight stabilization has also been reported for 5-amino-hexyl-substituted pyrimidines and has been attributed to shielding of the negative phosphate charges in unmodified hybrid duplexes (**45**). Interestingly, in another sequence, substitution of five thymidines with an analog containing a six-atom, amino-ethyl-3-acrylimido modifier at the 5 position of dU (**84**) (Glen Research, Sterling, VA) resulted in an increase in T_M of 1.2°C per substitution (M. Manoharan, unpublished results). Perhaps the acrylimido group contributes to stacking in a manner similar to the propyne substitution.

In contrast to the stabilizing or neutral effect of substituents at the 5 position, substitution at the 6 position (**85–87**) was very destabilizing. This destabilization is most likely related to the inability of these nucleosides to adopt the anti conformation due to the bulk of the substituent at position 6 (**20**).

Figure 4B summarizes the effect of combinations of 5 and 2′ substituents. In all cases the effects were roughly additive. Combination of two stabilizing modifications such as 2′ fluoro and 5-propynyl (**88**) resulted in a very stable hybrid. When stabilizing and destabilizing modifications were combined, for example, 2′-*O*-methoxy-ethyl with 5-methoxy substitution, the effect on duplex stability was essentially neutral.

Effect of other pyrimidine heterocycle modifications. Tables 9 and 10 report ΔT_M values for other pyrimidine modifications. Substitution of O4 or O2 of 2′-*O*-methyl U (**92–94**) resulted in extreme duplex destabilization (Fig. 4C). This is likely due to the

Table 8. Effect of 5 or 6 pyrimidine substitution on T_M



#	Modification -R1	-R2	-R3	ΔT_M per mod (parent is DNA) seq1	seq2	seq3	seq4	seq5	seq6	reference
(1)	-H	-H	-H	-0.6	+3.2	-0.2	-0.4	+0.1	-0.3	20
(78)	-F	-H	-H	-0.2						20
(79)	-Br	-H	-H	+0.3						20
(80)	-I	-H	-H	-0.1						20
(81)	-C≡C-CH_3	-H	-H	+0.9	+2.6	+1.7	+2.1	+2.6		5,42,102, P. Martin, unpublished results
(82)	-$C_3H_6NH_2$	-H	-H		+0.7			-0.2		P. Martin, unpublished results
(83)	-CH_2-O-C_2H_4-O-CH_3	-H	-H	-1.0	-2.2	-1.6		-1.2		P. Martin, unpublished results
(85)	-H	-CH_3	-H	-3.9						20
(86)	-CH_3	-CH_3	-H	-3.3						20
(87)	5-6 propyl bridge[1]		-H	-2.7	-1.3					Y. S. Sanghvi, unpublished results
(88)	-C≡C-CH_3	-H	-P		+2.6	+3.1				O. Acevedo, unpublished results
(89)	-C≡C-CH_3	-H	-O-C_2H_4-O-CH_3		+2.1	+2.3		+3.6		103
(90)	-O-CH_3	-H	-O-C_2H_4-O-CH_3	-0.2			+0.3	+0.4		P. Martin, unpublished results
(91)	-O-C≡C-CH_3	-H	-CH_3		-2.2		-1.0	-0.6		C. Schmit, unpublished results

[1]See Figure 4A for structure.

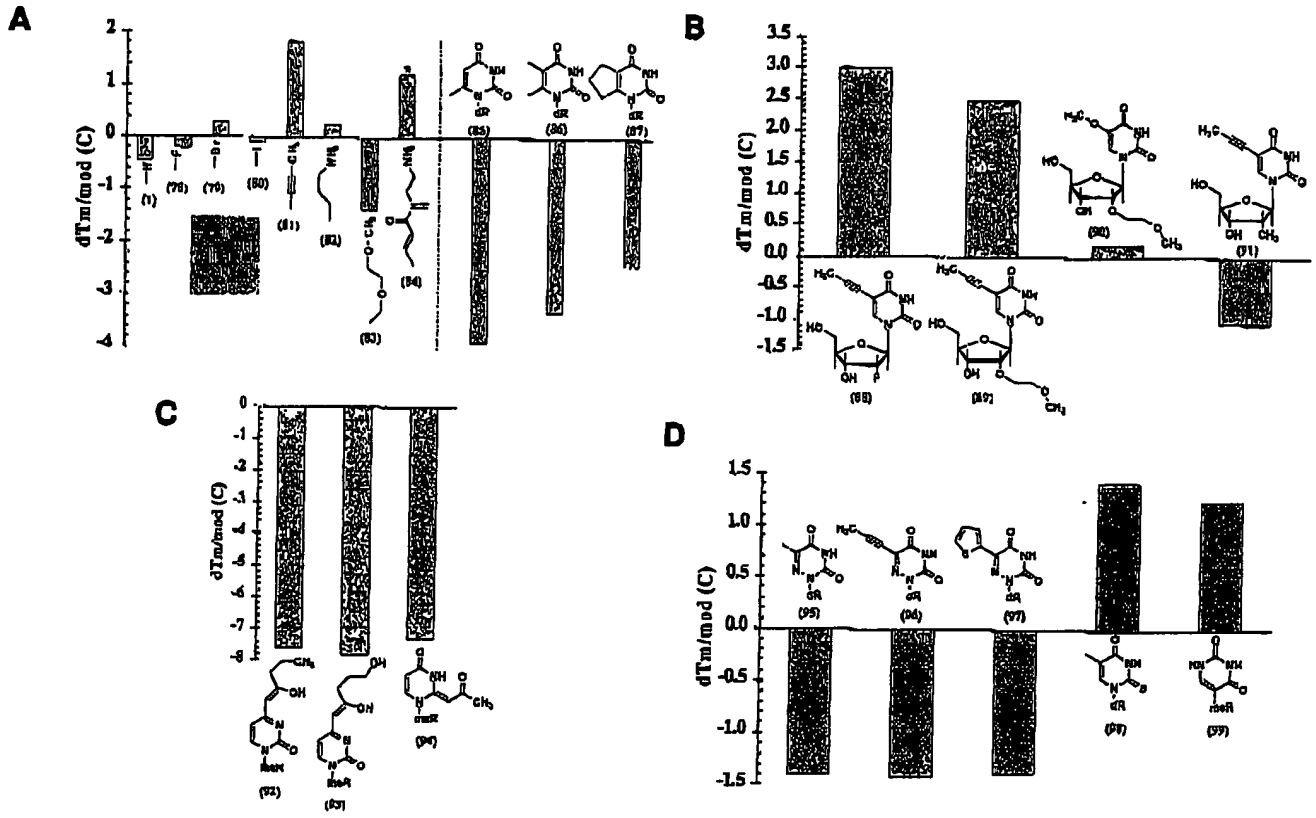
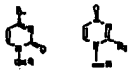


Figure 4. Average ΔT_M (°C) per substitution for oligonucleotides containing modified heterocycles. (A) 5- and 6-substituted thymidines; (B) 5 substitution combined with 2′ substitution; (C) substitution at O4 or O2 on 2′-*O*-methyl U; (D) 6 aza T analogs, 2 thio T and 2′-*O*-methyl pseudo uridine. The modification marked with an asterisk was evaluated in the sequence CtCGtACCmCCGtCC.

Table 9. Effect of substitution of O4 or O2 of 2′-*O*-methyl uridine on T_M



mod #	Modification	ΔT_M per mod (paired to DNA) seq1	seq2	seq3	seq4	seq5	seq6	ref.
(92)	R1 = -C(OH)-CH-C$_2$H$_5$		-7.6					46
(93)	R1 = -C(OH)-CH-C$_3$H$_7$-OH		-7.8					46
(94)	R1 = -CH$_2$-CO-CH$_3$	-0.6	-10.2					46

Table 10. Effect of modified pyrimidines on T_M

mod #	Modification[1]	ΔT_M per mod (paired to DNA) seq1	seq2	seq3	seq4	seq5	seq6	reference
(95)	6 aza T	+1.4						20
(96)	6 aza, 5 propynyl dU		-2.3	-1.2				104
(97)	6 aza, 5 thiazyl dU		-3.2	-1.9	-1.0			104
(98)	2 thio T	+0.9	+0.5		+1.7			E. Swayze, unpublished results
(99)	2′-O-methyl-pseudo U	+1.6	+0.3	+1.0	+1.3			50

[1]Structures of these modified nucleosides are shown in Figure 4D.

fact that these modifications remove hydrogen bonding sites in the heterocycle (46).

Substitution of T with 6-aza T (95) was also destabilizing (Fig. 4D). We speculate this destabilization is due to decreased H-bonding because the reduced pK_a for 6-aza T, compared to T, shifts the nucleoside toward the enol tautomer (47–49). In contrast to the results in Figure 4B, addition of a 5-propynyl group to 6-aza T (96) did not improve RNA binding affinity.

Figure 4D also plots data for 2-thio T (98) and 2′-*O*-methyl pseudo U (99). 2-thio-T resulted in an average increase of T_M of +1.4°C per substitution (Fig. 4D). This may be due to a tendency of the 2-thio nucleoside to adopt a C3′ *endo* sugar conformation (E. Swayze, unpublished results). This modification also improved binding to DNA targets suggesting improved stacking also contributes to duplex stability. The stabilizing effect of 2′-*O*-methyl pseudo U (99) was greater than that of 2′-*O*-methyl U (6) suggesting that the modified heterocycle itself also contributes to enhanced duplex stability (50).

Effect of purine heterocycle modifications. Although the series of oligonucleotides used in this study contained modifications only on pyrimidine residues, it is important to note that modifications of the purine heterocycle have also been described which result in improved hybrid stability. Among the most stabilizing purine modifications are the 7-halo-7-deaza purines (51,52) and the 7-propyne-7-deaza purines (53). The likely cause of increased duplex stability for these modifications is increased stacking of the modified purine rings.

Another modification that stabilizes the duplex is 2-amino-adenosine (2,6-diamino-purine). The amino group allows an additional H-bond to form with U and results in an increase in T_M of ~1°C per substitution (54, E.Lesnik, unpublished results).

Backbone modifications

The effect of non-phosphorus containing backbone modifications. The unmodified phosphodiester backbone contains five bonds and four atoms (-O-PO_2-O-CH_2-) between the five-membered rings of adjacent residues. Several modifications were tested in which these four atoms were replaced with a non-phosphorous containing backbone (Table 11). Replacement of the phosphate backbone with four CH_2 groups (**100**) severely destabilized the duplex (Fig. 5A). Flexible glycol and ether linkages (**101–103**) were also very destabilizing. When C=C double bonds (**105–107**) or C≡C triple bonds (**108–111**) were incorporated into the backbone, destabilization was less pronounced but in no case did oligonucleotides with an all carbon backbone hybridize to complementary RNA with the same affinity as unmodified DNA (Fig. 5A). In contrast to these all carbon backbones, the thioformacetal backbone (-S-CH_2-O-CH_2-) increased T_M 0.8°C per substitution (55). This stabilization was attributed to the compatibility of the backbone with the conformation of the DNA:RNA duplex due to a shift of the sugar conformation toward C3′ *endo* because of the reduced electronegativity of sulfur compared to oxygen (54).

Figure 5B plots average ΔT_M values for oligonucleotides containing unsubstituted urea (**112**), carbamate (**118** and **123**) and amide (**125, 128–129, 139–142**) linkages. Three-atom (**143–144**) and five-atom (**145**) amide linkages were destabilizing. Urea, carbamate and five of the four-atom amide backbones were also destabilizing. Only two amides did not destabilize the duplex, both of which had the amide moiety located in the middle position. They have been termed amide 3 (**129**) and amide 4 (**139**). Modeling studies of the structures in Figure 5B suggest that the backbone conformers for these two amide modifications most closely approach backbone conformations in a hybrid duplex (56,57). Thus, the stability of these modifications is likely due to a tendency of the backbone in the single strand to preorganize in conformations favorable for duplex formation (58). Apparently the less flexible urea and carbamate backbones and the destabilizing amide backbones prefer backbone conformations unfavorable for duplex formation. The beneficial effect of a rigid bond in the middle position (as in amides 3 and 4) was also observed for a *trans* C=C double bond (**105**) which was the least destabilizing of the all carbon backbones (Fig. 5B). A single oligonucleotide uniformly modified with amide 3 was also investigated (**130**). Its T_M was slighdly lower than that of the unmodified DNA control suggesting that the flexibility of intervening phosphates is required to obtain improved hybridization compared to natural DNA.

To explore the effect of conformational rigidity in the backbone on duplex stability further, four analogs of amides 3 and 4 were tested with an additional bond between the 3′ methylene group and C2′ of the deoxyribose (**162–165**). Structures of these analogs are given in Figure 5C. ΔT_M values are listed in Table 12. As is seen in Figure 5D, all of these constrained structures were much more destabilizing than the parent amides.

Data for more four-atom, non-phosphorous backbones are summarized in Figure 5E. Among these amine, hydroxylamine and hydrazino backbones, only two were stabilizing. These were the methylene(methylimino) or MMI (**148**) and the dimethylhydrazino (MDH) (**157**). Stabilization by the MMI backbone has been attributed to the fact that the 3′ methylene group of the MMI linkage induces a C3′ *endo* sugar conformation in the sugar 5′ to the linkage (59).

Table 11. Effect of non-phosphorous backbones on T_M

mod #	Backbone (•W-X-Y-Z•)	ΔT_M per mod (parent is DNA)						reference
		seq1	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(100)	-CH_2-CH_2-CH_2-CH_2-		-4.2	-3.6	-5.9	-3.1		57,103,106
(101)	-CH_2-CH_2-CH_2-O-				NC[1]			107
(102)	-O-CH_2-CH_2-O-				-3.3			107
(103)	-S-CH_2-CH_2-O-				NC[1]			K. Teng, unpublished results
(104)	-CH_2-CO-CH_2-CH_2-				-2.8	-2.1		57,106
(105)	-CH_2-CH=CH-CH_2- *(trans)*				-0.6	-1.0		108
(106)	-CH_2-CH=CH-CH_2- *(cis)*				-1.3	-1.5		108
(107)	-CH=CH-CH_2-CH_2-				-3.2	-3.3		P. von Matt, unpublished results
(108)	-O-CH_2-C≡C-				-3.1	-1.8		109
(109)	-S-CH_2-C≡C-					-4.6		109
(110)	-CH2-CH(OCH3)-O-C-(R)				-2.3	-3.1		109
(111)	-CH2-CH(OCH3)-O-C-(S)				-0.5	-3.6		109
(112)	-NH-CO-NH-CH_2-		-4.6	-3.0	NC[1]	-3.0		110
(113)	-NH-CO-NCH3-CH_2-		-2.9	-3.4	-6.4	-3.7		110
(114)	-NH-CO-NC3H7-CH_2-		-3.9	-4.2		-3.2		110
(115)	-NCH3-CO-NH-CH_2-		-7.7	-5.2		-3.7		110
(116)	-NH-CO-NC3H7-CH_2-		-3.3			-2.9		110
(117)	-NCH3-CO-NCH3-CH_2-					-5.2		110
(118)	-O-CO-NH-CH_2-		-3.8	-3.2	-4.8	-2.9		111
(119)	-O-CO-NCH3-CH_2-		-3.4	-2.3	-4.0	-2.0		111
(120)	-O-CO-NC3H7-CH_2-		-2.5					111
(121)	-O-CO-NC3H7-CH_2-		-1.2	-2.3	-3.4	-1.2		111
(122)	-S-CO-NCH3-CH_2-				-6.7	-4.0		112
(123)	-NH-CO-O-CH_2-		-7.0	-5.0	NC[1]	-3.8		111
(124)	-NCH3-CO-O-CH_2-		-5.6	-4.8	NC[1]	-3.9		111
(125)	-NH-CO-CH_2-CH_2-		-4.2	-2.8	-3.5	-2.7		113
(126)	-NCH3-CO-CH_2-CH_2-		-3.7	-2.9	-3.8	-2.4		113
(127)	-NC3H7-CO-CH_2-CH_2-		-2.5	-3.1	-5.0	-3.9		113
(128)	-CH_2-CH_2-NH-CO-		-1.3	-1.8	-2.8	-0.5		114
(129)	-CH_2-CO-NH-CH_2-		+0.9	+0.5	-0.1	+0.4	+0.8[4]	56,115
(130)	-CH_2-CO-NH-CH_2- *uniform*[2]						-0.4[4]	K.-H. Altmann, unpublished results
(131)	-CH_2-CO-NCH3-CH_2-	+1.0	-0.2			-1.1		56
(132)	-CH_2-CO-NC3H7-CH_2-	+0.9	-0.2	-0.2		-0.4		56
(133)	-CH_2-CO-N($C_2H_4OC_2H_4OC_2H_5$)-CH_2-	-0.0	-0.3	-0.3		-0.7		116
(134)	-CH_2-CO-N(C_2H_5)-CH_2-					-0.7		116
(135)	-CH_2-CO-N(CH_2-CH_2-CH_2)-CH_2-			NC		-3.5		116
(136)	-CH_2-CO-N(C_2H_4-O-CH_2-O-CH_2-O-CH_3)-CH_2-			-1.3		-2.0		116
(137)	-CH_2-CO-N(C_2H_4-N(CH_3)$_2$)-CH_2-			-1.0				P. von Matt, unpublished results
(138)	-CH_2-CO-NH-CH_2- *(with 5 propyne)*[3]				+0.8[3]	+0.4[3]		A. De Mesmaeker, unpublished results
(139)	-CH_2-NH-CO-CH_2-		-0.5	-0.3	+0.6	-0.8		117
(140)	-CO-NH-CH_2-CH_2-		-3.8	-3.3		-3.4		118
(141)	-O-CH_2-CO-NH- (carbo sugar on bottom)				NC[1]	-4.5		K. H. Altmann, unpublished results
(142)	-CH_2-CH_2-CO-NH- (carbo sugar on both)				-2.8			K. H. Altmann, unpublished results
(143)	-CH_2-NH-CO-					-1.8		119
(144)	-CO-NH-CH_2-					-3.0		119
(145)	-CH_2-CH_2-CO-NH-CH_2-				-3.0	-1.1		119
(146)	-CH=N-O-CH_2-		-3.1					120
(147)	-CH_2-NH-O-CH_2-		-0.5	-1.0	-1.2			120
(148)	-CH_2-N(CH_3)-O-CH_2-		-1.3	-0.2	+0.1		+1.5[4]	121
(149)	-CH_2-N(C_2H_5)-O-CH_2-				-0.5			122
(150)	-CH_2-N(C_3H_7)-O-CH_2-				-0.7			122
(151)	-CH_2-N(CH_2-CH(CH_3)$_2$)-O-CH_2-				-1.8			122
(152)	-CH_2-N(C_2H_4-O-CH_3)-O-CH_2-				-0.5			122
(153)	-CH_2-N(CH_2-NH)-O-CH_2-				-0.8			122
(154)	-CH_2-N(C_2H_4-N(CH_3)$_2$)-O-CH_2-				-1.0			122
(155)	-CH_2-N(CH_2-C_2H_3)-O-CH_2-		-0.0	-1.2	-2.2			122
(156)	-CH_2-N(-CH_2-C_2H_3)-O-CH_2-				-5.2			122
(157)	-CH_2-N(CH_3)-N(CH_3)-CH_2-		+1.6	-0.1	+0.2			123
(158)	-CH_2-O-N(CH_3)-CH_2-			-1.3	-2.0			124
(159)	-CH_2-N(CH_3)-CH_2-CH_2-		-2.5	-2.1	-2.6			125
(160)	-CH_2-CH_2-N(CH_3)-CH_2-		-3.8	-3.3	-4.4			125
(161)	-O-N(CH_3)-CH_2-CH_2-		-1.2	-0.9	-0.9	-		126

[1]NC, non-cooperative transition.

[2]This oligonucleotide contained modified TT and TC dimers.

[3]This oligonucleotide contained an amide backbone at all 14 positions with no intervening phosphates. The heterocycles were T and 5-methyl dC. ΔT_M is relative to a reference DNA oligo containing T and 5-methyl dC.

[4]In addition to the amide backbone, these oligonucleotides contained a 5-propyne substitution on the T 3′ to each backbone substitution.

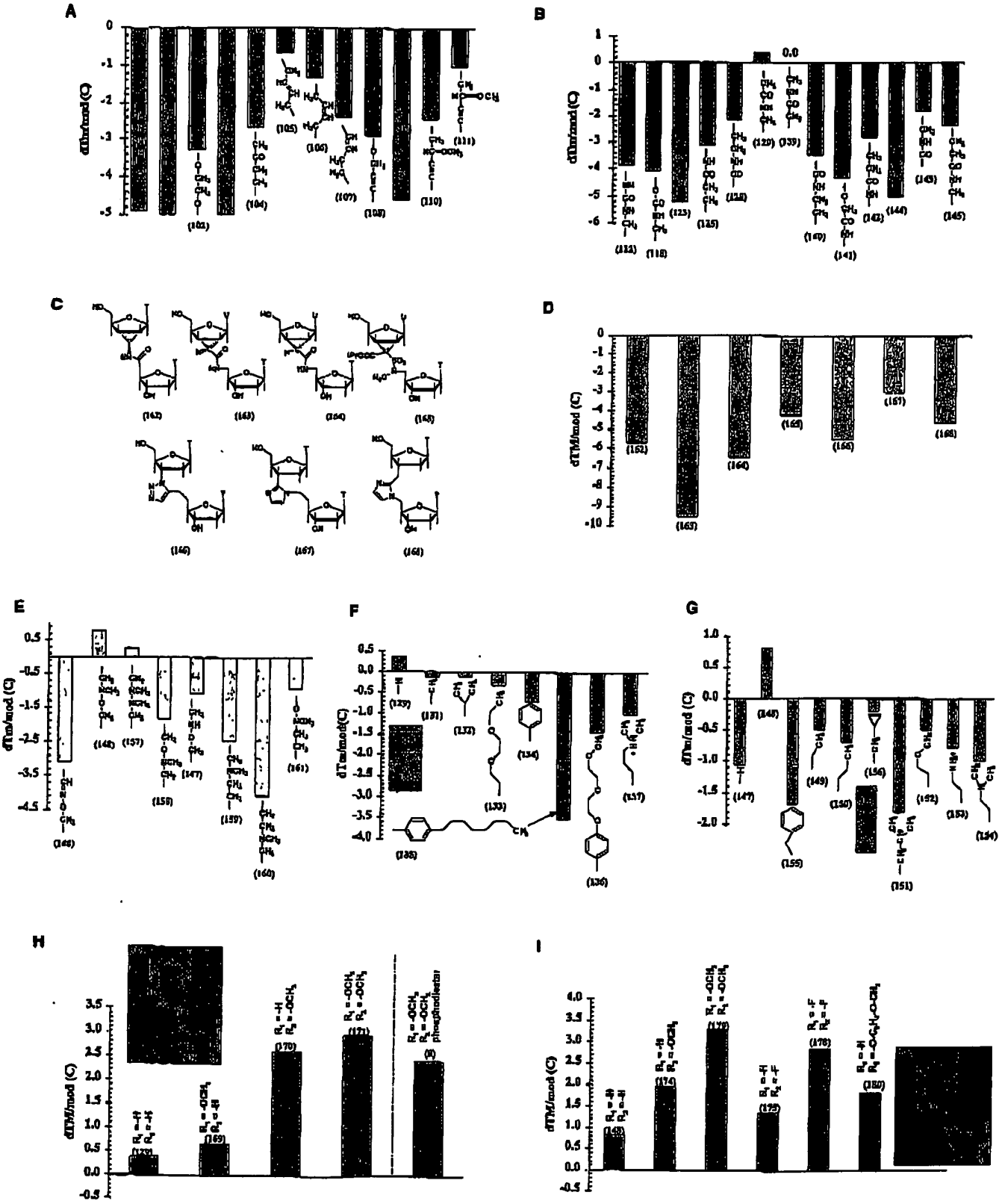


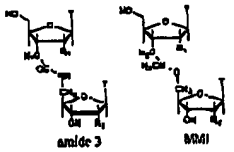
Figure 5. Average ΔT_M (°C) per substitution for oligonucleotides containing non-phosphorous backbone modifications. (**A**) Glycols, ethers and all carbon backbones; (**B**) urea, carbamate and amide backbones; (**C**) structures of cyclic backbone substitutions; (**D**) cyclic backbone substitutions; (**E**) amines, hydroxylamines and hydrazino backbones; (**F**) *N*-substituted amide 3 backbones; (**G**) *N*-substituted MMI backbones; (**H**) 2′-substituted amide 3 modifications; (**I**) 2′-substituted MMI modifications. Modifications marked with an asterisk resulted in a non-cooperative transition for the only sequence studied.

Table 12. Effect of cyclic backbone substitutions on T_M

mod #	ΔT_M per mod (parent is DNA)						ref.
	seq1	seq3	seq4	seq4	seq5	seq6	
(165)			-5.6	NC[2]			127
(163)				-4.2[3]			128
(164)				-9.5[4]			128
(165)				-6.4[4]			128
(166)				-0.1	-2.3		129
(167)				-3.7[5]	-1.0		129
(168)				-4.6[5]			129

[1]Structures of these backbone modifications are shown in Figure 5C.
[2]NC, non-cooperative transition.
[3]These oligonucleotides contained only three backbone modification, at positions 4–5, 8–9 and 12–13.
[4]This oligonucleotide contained only one backbone modification, at position 8–9.
[5]This oligonucleotide contained only two backbone modifications, at positions 6–7 and 12–13.

Table 13. Effect of 2′ substitution on T_M of amide 3 or MMI-modified oligonucleotides



mod #	Backbone	R1	R2	ΔT_M per mod (parent is DNA)						reference
				seq1	seq2	seq3	seq4	seq5	seq6	
(169)	amide 3	-O-CH_3	-H				+0.9	+0.0		130
(170)	amide 3	-H	-O-CH_3				+2.0	+2.1	+2.1[1]	130
(171)	amide 3	-O-CH_3	-O-CH_3				-3.0	+2.6		130
(172)	amide 3	-O-H	-H					+1.2		130
(173)	N-phenyl amide 3[2]	-H	-O-CH_3	-1.7						A. Waldner, unpublished results
(174)	MMI	-H	-O-CH_3		+0.9	-1.7	+2.3			131,132
(175)	MMI	-H	-F		-0.3	+1.0	+1.8			131,132
(176)	MMI	-O-CH_3	-F		+1.6	+2.5	+3.2			131,132
(177)	MMI	-F	-O-CH_3		+2.0	+3.0	+3.8			131,132
(178)	MMI	-F	-F		+1.3	+2.2	+3.4			131,132
(179)	MMI	-O-CH_3	-O-CH_3		+1.9	+2.8	+3.8			131,132
(180)	MMI	-H	-O-CH_2-CH_2-O-CH_3		+1.0	+1.6	+2.1			Y. S. Sanghvi, unpublished results

[1]This oligonucleotide contained T and 5-methyl C heterocycles.
[2]In addition to the indicated 2′ substitutions, this oligonucleotide contained an *N*-phenyl substitution in the amide backbone.

In an attempt to identify analogs of amide 3 or MMI with improved hybridization properties, several *N*-substituted variants were investigated. Results for *N*-substituted amide 3 (**131–137**) and *N*-substituted MMI (**147–156**) are summarized in Figure 5F and G, respectively. Although small substituents on the nitrogen of amide 3 were tolerated, none improved hybridization compared to amide 3 itself (**129**) and large substituents were very destabilizing (Fig. 5F). For the methyleneimino backbone, only the *N*-methyl analog (**148**) was stabilizing (Fig. 5G). All other nitrogen substituents were destabilizing.

Figure 5H and I and Table 13 summarize the effects of adding 2′ substitutions to amide 3 (**129**) and MMI (**148**), respectively. For both backbones, addition of a 2′-*O*-methyl group on the lower sugar (the sugar 3′ of the modified linkage) (**170, 174**) greatly stabilized the duplex and 2′-*O*-methyl substitution on both sugars of the modified backbone (**171, 179**) stabilized even more than 2′-*O*-methyl substitutions on a phosphate diester backbone (**8**). Similar effects were observed for 2′-fluoro (**175–178**) and 2′-*O*-methoxy-ethyl (**180**) substitution. This stabilization was explained by the effect of the backbone and the 2′ substituents on the sugar pucker (60). For the bis-deoxy MMI modification (**148**) the conformational analysis indicated 68 and 31% northern conformation for the upper and lower sugar, respectively, compared to ~30% northern for sugars in unmodified DNA. Addition of a 2′-*O*-methyl group to the lower sugar of the MMI linked dimer units (**174**) shifted the conformational equilibrium to ~65% northern conformation for both sugars and resulted in a significant increase in T_M. Addition of a second 2′-*O*-methyl on the upper sugar (**179**) increased the fraction of C3′ *endo* conformation to 95 and 76% for the upper and lower sugar, respectively, and stabilized the duplex even further. Thus the high stability of modified DNA:RNA duplexes incorporating intrinsically favorable backbone modifications in combination with electronegative 2′ substituents appears to be strongly correlated with the conformational equilibria of the sugars.

Table 12 and Figure 5C and D report T_M data for backbones containing triazole (**166**) and imidazole (**167–168**) heterocycles. All of these cyclic backbones were destabilizing.

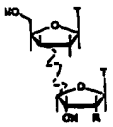
Modified backbones containing phosphorous. Among oligonucleotide modifications used for antisense applications, those that have been tested most extensively are phosphate-modified backbones. These include phosphorothioates (61), phosphoramidates (62–64) and methyl phosphonates (65) in which one of the non-bridging phosphate oxygens has been replaced by sulfur, -NHR or -CH_3, respectively. All of these modifications result in reduced hybrid stability. It has been suggested that this destabilization is caused by diastereoisomerism due to chirality at phosphorous, however, phosphorodithioates, which contain an achiral phosphorous atom, also destabilize the duplex (66–68).

In contrast, substitution of the bridging 3′-oxygen with NH (N3′→P5′ phosphoramidates) resulted in very stable duplexes with T_M increases of ~2°C per substitution (69). Even greater stabilization of 4°C per substitution was reported for 2′-fluoro, N3′→P5′ phosphoramidate oligonucleotides (70). These stabilizations, which are some of the largest reported to date have been attributed to the tendency of the sugar moieties to adopt a C3′ *endo* conformation when the 3′-O is replaced with 3′-NH (71).

ΔT_M data for oligonucleotides containing other types of phosphorous modifications are reported in Tables 14 and 15. Averaged data are plotted in Figure 6. Both isomers of an ethyl phosphinate moiety (**181–182**) were destabilizing, as was the free phosphinate (**184**) (Fig. 6A). Shorter, three-atom phosphinates (**189–190**) were also destabilizing. Although addition of a 2′-*O*-methyl group to the lower sugar of the four-atom ethyl phosphinate modified dimer units (**185–188**) improved hybridization, these modifications were still destabilizing. Because the phosphinate backbone modified oligonucleotides hybridized to DNA much more poorly than to RNA, it was suggested that the lack of an electronegative group at C3′ likely favors a northern sugar pucker (72). This is supported by the observation that replacement of the 3′CH_2 with a more electronegative CHF (**191–192**) reduced duplex stability even further.

Data for other phosphorous containing backbones are plotted in Figure 6B. Thio-phosphate (**193**), which has been widely used for antisense applications, reduced T_M –0.7°C per substitution. Averaged over several uniformly modified sequences, mixed diastereoisomers of thiophosphates reduced T_M by ~0.5°C per substitution (E. Lesnik, unpublished results). Phosphine oxide

Table 14. Effect of phosphinate substitutions on T_M



mod #	backbone	stereochemistry at phosphorous[2]	R	ΔT_M per mod (parent is DNA)						reference
				seq1	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(181)	-CH_2-PO(O-C_2H_5)-CH_2-CH_2-	1	-H		-2.2	-1.8		-3.3		72
(182)	-CH_2-PO(O-C_2H_5)-CH_2-CH_2-	2	-H		-1.8	-1.3		-2.8		72
(183)	-CH_2-PO(O-C_2H_5)-CH_2-CH_2-	mix	-H		-1.6	-1.5		-1.3		72
(184)	-CH_2-PO-CH_2-CH_2-	–	-H		-2.4	-1.6	-1.8	-2.8		72
(185)	-CH_2-PO(O-C_2H_5)-CH_2-CH_2-	1	-OCH_3					-1.4		S. Collingwood, unpub.
(186)	-CH_2-PO(O-C_2H_5)-CH_2-CH_2-	2	-OCH_3				-0.3	-0.2		S. Collingwood, unpub.
(187)	-CH_2-PO(O-C_2H_5)-CH_2-CH_2-	mix	-OCH_3				-0.4	-1.0		S. Collingwood, unpub.
(188)	-CH_2-PO-CH_2-CH_2-	–	-OCH_3				-0.4	-1.7		S. Collingwood, unpub.
(189)	-CH_2-PO(O-C_2H_5)-CH_2-	1	-H				-3.2	-2.9		S. Collingwood, unpub.
(190)	-CH_2-PO(O-C_2H_5)-CH_2-	2	-H				-3.6	-4.3		S. Collingwood, unpub.
(191)	-CFH-PO(O-C_2H_5)-CH_2-CH_2- (*R*)[1]	mix	-H					-3.5		S. Collingwood, unpub.
(192)	-CFH-PO(O-C_2H_5)-CH_2-CH_2- (*S*)[2]	mix	-H				-2.1	-2.4		S. Collingwood, unpub.

[1]Stereochemistry at the site of fluoro substitution was *R*.
[2]Stereochemistry at the site of fluoro substitution was *S*.
[3]Isomers 1 and 2 represent the two diastereoisometrically pure isomers. Absolute stereochemistry, at phosphorous, of the isomers has not been determined.

Table 15. Effect of phosphate backbone modification on T_M

mod #	backbone	stereochemistry at phosphorous[1]	ΔT_M per mod (parent is DNA)						reference
			seq1	*seq2*	*seq3*	*seq4*	*seq5*	*seq6*	
(193)	-O-PO$_2$-O-CH_2-	mix					-1.0	-0.7	D. Hüsken, unpublished results
(194)	-CH_2-PO(CH_3)-CH_2-CH_2-	mix				-3.9	-2.4		S. Collingwood, unpublished results
(195)	-CH_2-PO(CH_3)-CH_2-CH_2-	1					-3.5		S. Collingwood, unpublished results
(196)	-CH_2-O-PO$_2$-O-CH_2-	–		-1.8	-1.5	-1.8			133
(197)	-O-CH_2-CH_2-O-PO$_2$-O-CH_2-	–					-3.8		P. Martin, unpublished results

[1]Isomer 1 represents a diastereoisometrically pure isomer. Absolute stereochemistry, at phosphorus, has not been determined.

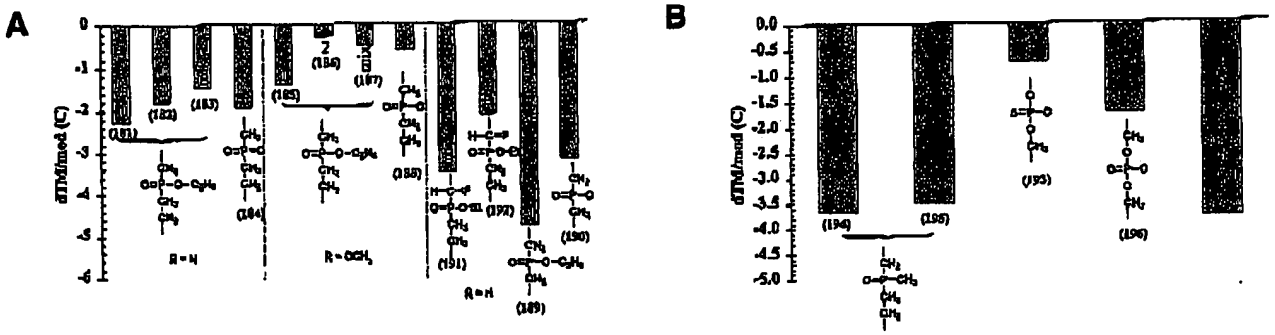


Figure 6. Average ΔT_M (°C) per substitution for oligonucleotides containing modified phosphate backbones. (A) Phosphinate analogs and (B) phosphate and phosphine oxide backbones.

modifications (194–195) and longer phosphate backbones (196–197) were very destabilizing.

Other neutral backbones. In addition to the modifications mentioned above, there are two interesting modifications that could not be studied in the partially modified sequences of Table 1 because the synthetic strategies used for these modifications could not easily be combined with DNA phosphoramidite chemistry. These modifications are the phosphoryl linked morpholino backbone (199) of Summerton and Weller (73–75) and the polyamide backbone called PNA (198) (17,19). Structures and ΔT_M values for these modifications are given in Figure 7. The increased hybrid stability observed for these modifications is likely due to their neutrality and probably reflects a tendency of the single strands to adopt conformations favorable for duplex formation.

Specificity of hybridization. For antisense applications, high specificity of Watson–Crick binding is as important as high affinity of hybridization. For evaluation of hybridization specificity, T_M was measured for *seq2* against RNA targets containing mismatched nucleotides (C, G or U) opposite the modified T. T_M with the matched target was compared to T_M with the mismatched

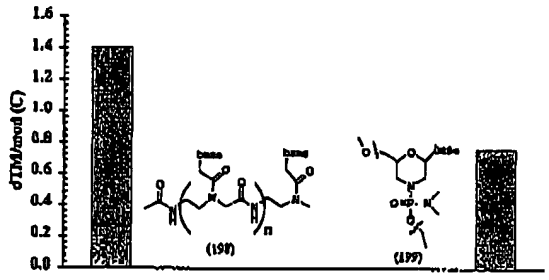


Figure 7. Structure and ΔT_M per substitution for the PNA (198) and morpholino (199) backbones used in this study. For PNA, ΔT_M per substitution was averaged over the sequences TGTACGTCACAACTA, GCACAGCC, TATTCCGTCATCGCTCCTCA, TTTAGGATTCGTGCTCATGG, GCCTTTCGCGACCCAACACT, CGCTCAAGTCCCATCGACCT, TAATGCGTACCATATGC, CGACTATGCAAGTAC, CGCTTGGCAGTCTC. For morpholino, ΔT_M was measured in a single sequence, UCUGAGUAGCAGAGGAGCUC.

targets. For all modifications that resulted in increased or only a slightly decreased duplex stability (not more than −1°C per substitution), specificity of the modified oligonucleotide was never worse than that of the unmodified DNA parent. The only modifications that showed poor specificity were those that resulted in sizable destabilization. These destabilizing modifications likely lead to distortions in duplex structure that cause disruption of base pairing at the site of modification and thus loss of Watson–Crick base pair specificity.

DISCUSSION

Analysis of the results presented above reveals four approaches that can be used to modify DNA for improved hybridization to RNA targets: (i) preorganize the sugars and phosphates of the DNA single strand into conformations favorable for hybrid formation, (ii) improve stacking by adding a polarizable group to the heterocycle, (iii) increase the number of H-bonds for an A–U pair and (iv) neutralize the backbone charge. Examples of each of these will be discussed below.

Modifications that shift the sugar conformation toward the northern pucker

Sugars in DNA:RNA hybrid duplexes frequently adopt a C3′ *endo* conformation. Thus modifications that shift the conformational equilibrium of the sugar moieties in the single strand toward this conformation should preorganize the antisense strand for binding to RNA. Several types of modifications reported above shifted the sugar toward a C3′ *endo* conformation. Substitution with an electronegative atom at the 2′ position [e.g. 2′-fluoro (**4–5**) (Fig. 1A) or 2′-OR (**6–15, 28–49**) (Fig. 1A, C and D)] resulted in a shift towards the northern conformation and, in general, increased T_M. Large 2′-*O*-alkyl substituents, however, were not well tolerated, presumably because of steric interference by the flexible alkyl chain with other parts of the duplex. However, large 2′-*O* substituents were tolerated if they contained the ethylene glycol motif (**35, 37–49**) (Fig. 1C and D). Apparently the *gauche* effect of the oxygen γ to the 2′ oxygen results in a configuration of the side chain favorable for duplex formation.

Shift of the sugar conformation towards a northern pucker and an increased T_M were also observed for modifications in which the 3′-oxygen was replaced with a non-electronegative group such as CH_2 in MMI (**148**) (Fig. 5E) or amide 3 (**129**) (Fig. 5B), with S in the thioformacetal backbone (-S-CH_2-O-CH_2-) (**55**), or with NH in the N3′→P5′ phosphoramidate backbone (69). Thus an electronegative group at the 2′ position or a non-electronegative group at the 3′ position was effective in shifting the sugar conformation and improving T_M. Although it seems clear that the presence of a less electronegative group than oxygen at C-3′ represents an important feature for modifications that enhance duplex stability, this characteristic is by no means sufficient to enhance RNA binding affinity. This is amply illustrated by a whole range of backbone modifications incorporating a CH_2 group attached to C-3′ which did not lead to increased DNA:RNA duplex stability (Figs 5 and 6). Among these are Benner's sulfone modified oligonucleotides (-CH_2-CH_2-SO_2-CH_2-) which generate an A-type pucker but did not improve binding to RNA because the single sequence for which RNA binding has been reported formed a stable hairpin (76,77).

Another approach to shift the sugar conformation toward a northern pucker involves the introduction of conformational constraints using a 4′–6′ methylene bridge in the carbocyclic nucleoside (**73**) (Fig. 3). A change in the sugar conformational equilibrium toward a northern pucker can also be induced by certain base modifications without alterations in the 2′-deoxyribose. Thus, 2-thio T (**98**) in combination with an unmodified sugar–phosphate backbone still resulted in a shift of the sugar pucker towards a northern conformation and increased T_M (Fig. 4D).

The beneficial effect of preorganization of the sugar–phosphate backbone is also observed in the conformationally restricted 1′–5′ anhydrohexitol oligonucleotides which exhibit substantially improved hybridization compared to unmodified analogs (78–80). In this context, it should also be noted that the importance of conformational preorganization of the sugar–phosphate backbone is most impressively demonstrated by Eschenmoser's work on homo-DNA and related hexose-based nucleic acids (81–84). The stability of (2,3-dideoxy-D-glucopyranose-based) homo-DNA duplexes far exceeds that of natural DNA/DNA duplexes; however, due to their particular conformational properties, these analogs do not bind to natural nucleic acids and, in fact, would not be predicted to do so (81,82,84). On the other hand, incorporation of flexible, glycerol-based nucleoside analogs into oligodeoxyribonucleotides reduced binding affinity for complementary DNA (and presumably also RNA) dramatically (84–86) and neither did glycerol-based DNA analogs form stable self-duplexes (84,86). These findings may be rationalized by a reduction in appropriate conformational preorganization (increased entropy) similar to that observed for many flexible backbone modifications (see below).

Modifications that preorganize the backbone in conformations favorable for hybrid duplex formation

In addition to shifting the sugar to a conformation favorable for hybridization, modifications can also be made that preorganize the internucleotide backbone part of the modified DNA into conformations favorable for duplex formation. These modifications do not necessarily have to limit the single strand to a single conformation; they simply increase the population of single strands in conformations favorable for duplex formation and reduce the population in conformations incompatible with duplex formation. Modifications reported above that did this successfully were amide-3 (**129**) (Fig. 5B), amide-4 (**139**) (Fig. 5B), MMI (**148**) (Fig. 5E) and MDH (**157**) (Fig. 5E).

Preorganization of the backbone can also be detrimental for hybridization. Many modifications tested were less flexible than the normal phosphate backbone and likely resulted in preorganization of the antisense single strand but resulted in destabilization of the duplex. Examples include the three-atom (**143–144, 189–190**) and five-atom (**145, 196**) linkages in Figures 5B and 6. The conformations favored by these backbones likely were incompatible with duplex formation and resulted in a decrease in T_M. Clearly just the right amount of preorganization in just the right place was required for improved hybridization to occur.

Modifications that improve stacking by adding a polarizable group to the heterocycle

Favorable stacking of the heterocyclic bases contributes much of the favorable enthalpy of duplex formation for nucleic acid duplexes (14,87). This favorable stacking is due primarily to favorable interactions between dipoles and induced dipoles in adjacent residues. Thus modifications to the heterocycle that improve these interactions are likely to stabilize the duplex. Some examples include substitution at the 5 position of pyrimidine with propyne (**81**) (Fig. 4A), amino-ethyl-3-acrylimido (**84**) (Fig. 4A) or methylthiazole (43), tricyclic dC analogs (44) and 7-modified-7-deaza-purines (51–53).

Modifications that increase the number of H-bonds

H-bonds in RNA duplexes contribute ~1 kcal/mol of favorable free energy (88). This correlates well with the increase in T_M reported above for 2,6-diamino purine which can form three hydrogen bonds with U. Thus addition of a Watson–Crick H-bond can improve duplex stability.

Modifications that neutralize the negative phosphate charge

It has long been known that charge repulsion between phosphates on opposite strands provides a significant unfavorable contribution to the free energy of duplex formation at physiological ionic strengths (89,90). Thus removal of the negative charge on one strand is expected to increase duplex stability at physiological ionic strength. Several modifications described above reduced the net charge on the oligonucleotide and reduced the dependence of T_M on ionic strength (19,27, S. Freier, unpublished results). Only some of these resulted in an increase in T_M because often the favorable effect of the neutral charge was offset by an unfavorable effect such as preorganization into a structure incompatible with duplex formation or increased flexibility of the internucleotide linkage.

Some of the greatest increases in stability were observed for the PNA (**198**) and morpholino (**199**) modifications (Fig. 7) which are no longer negatively charged but whose backbone conformations are still compatible with duplex formation. A second approach to charge neutralization is to add a positive charge to the oligonucleotide. This was done most effectively at the 2′ position by addition of a 2′-*O*-amino-alkyl group (**30–31**) (Fig. 1C) and at the 5 position of T by addition of an amino alkyl (**82**) or an amino-ethyl-3-acrylimido group (**84**) (Fig. 4A).

Effect of combinations of stabilizing features

We have listed above four approaches for improving duplex stability and have presented examples for each approach. It is clear, however, that for most stabilizing modifications, more than one of these factors contributes to improved hybridization. For example, the stabilizing effect of MMI is a combination of the shift toward C3′ *endo* caused by the 3′ CH_2, restricted backbone flexibility and the neutral charge. Similarly, the stabilizing effect of 2-thio-T is likely a combination of the shift of the sugar pucker toward C3′ *endo* and improved stacking.

All four of the factors listed above also play a role in hybridization properties of destabilizing modifications. Frequently, in fact, one factor may contribute favorably but it is outweighed by another factor with a very unfavorable effect. For example, the ethyl phosphinates (**181–183**) (Fig. 6A) have a neutral backbone and the 3′-CH_2 helps to drive the sugar toward a C3′ *endo* conformation. In spite of these effects, however, the modifications were very destabilizing, probably because this backbone did not easily adopt conformations consistent with duplex formation. Of the modifications reported above, most were, in fact, very destabilizing. Usually, when a molecule was modified to favorably affect one of the factors listed above, the other factors were unfavorably affected for a net negative effect. Thus, net favorable effects were rare and the success rate was low.

The most stable duplexes reported above were formed with oligonucleotides that contained two different types of modification. These include 2′-*O*-methyl MMI backbones (**179**) (Fig. 5I), 2′-*O*-methyl amide 3 backbones (**171**) (Fig. 5H), 2′-*O*-methyl, 2-amino-adenosine (91), 2′-fluoro-5-propynyl dU (**88**) (Fig. 4B) and the 2′-fluoro, N3′→P5′ phosphoramidate oligonucleotides (70). The high T_Ms were achieved because each of the two modifications fulfilled one of the principles outlined above and no principle was violated. Thus careful combination of stabilizing modifications can produce even more stable duplexes.

It is important to note at this point that duplex stability will also be significantly affected by the difference in solvation energy between the single strands and the duplex. The importance of this parameter has been addressed in some detail in a recent review article by Elgi (23). Unfortunately, due to a lack of structural information, it is impossible to assess the relevance of solvation effects for the modified DNA:RNA duplexes discussed in this paper in any meaningful fashion. We do feel, however, that interactions with solvent may play an important role in distinguishing the effects of simple alkyl and ethylene glycol-based 2′-*O*-substituent on RNA binding affinity (see Results: Sugar modifications).

In summary, we have tabulated above, T_M data for roughly 200 modifications that were incorporated into a single set of sequences. We also tried to include data for stabilizing modifications studied in other sequences. In spite of the large number of modifications tested, only relatively few structures that significantly stabilize DNA:RNA duplexes were identified. It appears that modified oligonucleotides with very high RNA binding affinity need to be constructed by the combination of two or more different types of modifications, each of which contributes favorably to one of the general factors outlined above.

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Nuclease Resistance and Antisense Activity of Modified Oligonucleotides Targeted to Ha-*ras**

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We have previously described structure-activity studies on a 17-mer uniform phosphorothioate antisense sequence targeted to human Ha-*ras*. In an effort to further improve the pharmacological properties of antisense oligonucleotides, structure-activity studies on this 17-mer sequence were expanded to examine both the effects of replacing phosphorothioate backbone linkages with phosphodiester linkages and the effects of incorporating various 2′-sugar modifications into phosphorothioate and phosphodiester oligonucleotides on oligonucleotide stability against nucleases *in vitro* and on antisense activity in cells. Replacement of three or more phosphorothioate linkages with phosphodiester linkages greatly compromised both nuclease resistance and antisense activity, and these effects correlated directly with the number of phosphodiester linkages incorporated into the oligonucleotide. However, substantial nuclease resistance, sufficient for obtaining potent antisense effects in cells, was conferred to phosphodiester oligonucleotides by incorporation of appropriate 2′-alkoxy sugar modifications. Nuclease stability and antisense activity imparted by these sugar modifications in phosphodiester backbones correlated with the size of the 2′-alkoxy substituent (pentoxy > propoxy > methoxy > deoxy). Furthermore, antisense activity mediated by oligonucleotides that exhibit partial resistance to nucleolytic degradation was dependent on both oligonucleotide concentration and the duration of oligonucleotide treatment.

Susceptibility of unmodified phosphodiester oligodeoxynucleotides to nucleolytic degradation by intracellular and extracellular nucleases has made them unattractive molecules for oligonucleotide therapeutics. Reports on the stability of unmodified oligodeoxynucleotides in biological fluids have demonstrated half lives for these molecules as short as 5 min in serum and 30 min in living cells (1–4). The primary mechanism of oligodeoxynucleotide degradation in serum has been reported to be 3′-exonuclease activity, whereas both endonuclease and exonuclease activity have been reported to play significant roles in the degradation of these molecules in cells (2–7).

To alleviate the problem of nucleolytic degradation, chemical modifications of the natural phosphodiester backbone have been introduced into oligonucleotides to increase their stability in biological systems (8, 9). The most commonly employed synthetic modification designed to reduce oligonucleotide sensitivity toward nucleases is the phosphorothioate analog, created by replacing one of the nonbridging oxygen atoms of the internucleotide linkage with sulfur (10). Stein and co-workers (11) have reported that the stability of phosphorothioate oligonucleotides against purified nucleases *in vitro* varies greatly depending on oligonucleotide sequence and the type of nuclease examined. Studies have also been performed demonstrating that uniform modification of oligonucleotides with nuclease-resistant linkages is not required to confer enhanced stability. For example, increased resistance to degradation *in vitro* can be achieved by substitution of one or more phosphodiester linkages at the 3′-end of an oligonucleotide with phosphorothioate modifications (2–5). Alternating phosphorothioate modifications with phosphodiester linkages has also been shown to increase the stability of these molecules against purified nucleases *in vitro* (5).

Despite the fact that phosphorothioate oligonucleotides display many attractive features, some potential limitations do exist with these compounds. For example, high concentrations of phosphorothioates have been shown to competitively inhibit a variety of nucleases and polymerases (6, 7, 12–14), interact with and potentially abrogate the activity of heparin-binding growth factors (13, 15), induce immune stimulatory effects in rodents (13, 16), cause complement activation and hypotension in monkeys, and induce clotting abnormalities in monkeys as a result of direct interactions with thrombin (17, 18). Although these potential limitations have not proven to be problematic in clinical trials to date, evaluation of novel oligonucleotide modifications that reduce phosphorothioate content but maintain stability against nucleolytic degradation is obviously warranted.

Enhanced nuclease stability of phosphodiester oligonucleotides containing modified nucleosides has been investigated with some success. Incorporation of α-anomers into oligonucleotides has been shown to dramatically increase their stability against nucleolytic degradation (19, 20). Significant enhancement of nuclease resistance has also been demonstrated in oligonucleotides that contain a methylene group in place of the oxygen in the ribose ring (21). Replacement of the 2′-sugar deoxy substituent with 2′-*O*-methyl and 2′-*O*-allyl modifications has been reported to increase oligonucleotide stability toward various nucleases under cell-free conditions (22, 23).

Cummins *et al.* have extended these studies by demonstrating that the sensitivity of a variety of 2′-alkoxy phosphodiester oligonucleotides toward snake venom phosphodiesterase under cell-free conditions is dependent on the size of the 2′-substituent with nuclease resistance correlating directly with 2′-alkoxy chain length (24). In addition, it has been reported that fluorescently labeled 2′-*O*-methyl and 2′-*O*-allyl modified phosphodiester oligonucleotides are detectable in mammalian cells for greater periods of time following microinjection, as com-

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The nucleotide sequences described here were obtained from the GenBank™ Data Bank with the accession number J00277 (Ha-ras).

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pared with fluorescently labeled unmodified oligodeoxynucleotides (25).

Despite the progress achieved investigating the stability of modified oligonucleotides toward nucleases under cell-free conditions, reports directly demonstrating a relationship between the level of nuclease resistance observed for a modified oligonucleotide under cell-free conditions and the degree of antisense activity obtained in cells are rare. Unfortunately, conclusions regarding the level and duration of antisense activity that will be obtained by a modified, "nuclease-resistant," oligonucleotide based on the extrapolation of results from cell-free nuclease assays can be misleading. One reason for this is that the level of nuclease activity that must be overcome by an effective antisense oligonucleotide prior to, during, and after cell uptake is unknown. Furthermore, different cell types and intracellular compartments contain different types of nucleases and levels of nuclease activity (26). Additionally, sequence and secondary structure can greatly affect the sensitivity of an oligonucleotide to nucleolytic degradation (24). Thus, it is essential to directly compare cell-free nuclease results with antisense effects in cells when drawing conclusions regarding the utility of a nuclease- resistant modification for the purpose of antisense exploitation.

We have previously described a 17-mer phosphorothioate antisense oligonucleotide targeted to the codon 12 region of mutant Ha-*ras* (GGC → GTC) that displays selective inhibition of mutant Ha-*ras* expression, relative to wild type, in cells (27). Additionally, structure-activity studies have been performed on this phosphorothioate in which various 2′-sugar modifications were evaluated for their ability to direct RNase H cleavage of the target mRNA *in vitro*, to affect target affinity, and to modulate antisense activity against the Ha-*ras* message in cells (28). In that report, it was demonstrated that antisense activity can be significantly enhanced through the use of certain 2′-sugar modifications that hybridize to complementary RNA with a relatively high affinity, relative to unmodified DNA, provided that the oligonucleotide is designed as a chimera in which the 2′-sugar-modified region of the oligonucleotide, which is unable to activate RNase H, is fused to an RNase H-sensitive deoxy gap region of the appropriate length.

We now describe a systematic study in which modified chimeric oligonucleotides were characterized for both their relative susceptibility to degradation by purified nucleases *in vitro* and their ability to elicit antisense effects in cells. The antisense target for these studies was again the Ha-*ras* oncogene containing a GGC → GTC point mutation at codon 12 (27). Our results indicate that replacement of as few as three phosphorothioate linkages with phosphodiester linkages in an oligonucleotide greatly compromises both nuclease resistance and antisense activity and that substantial nuclease resistance, sufficient for obtaining antisense activity in cells, can be conferred to phosphodiester oligonucleotides through the use of appropriate 2′-alkoxy modifications. Furthermore, our findings demonstrate that the antisense activity observed for oligonucleotides that exhibit partial resistance to nucleolytic degradation is dependent on both the employed oligonucleotide concentration as well as the duration of oligonucleotide treatment.

MATERIALS AND METHODS

Cells and Reagents—The human bladder carcinoma cell line T24 was obtained from the American Type Tissue Collection (Bethesda, MD). T24 cells were grown in McCoy's 5a medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2. This cell line contains and expresses oncogenic Ha-*ras* containing a homozygous point mutation at codon 12 (GGC → GTC), (29, 30). DOTMA:DOPE[1] (Lipofectin) solution (*N*-[1-(2,3-dioleyloxy-propyl]-*N,N,N*-triethylammonium chloride) was purchased from Life Technologies, Inc. (Gaithersburg, MD). Opti-MEM was purchased from Life Technologies, Inc. Snake venom phosphodiesterase was purchased from U.S. Biochemical Corp. S1 nuclease was purchased from Life Technologies, Inc.

Oligonucleotide Synthesis—2′-alkoxy and 2′-fluoro monomers were synthesized as described previously (31, 32). Synthesis of phosphorothioate and phosphodiester oligonucleotides (deoxy and 2′-modified) were performed using an Applied Biosystems 380B automated DNA synthesizer as described previously (27). Purification of oligonucleotide products was also as described previously (27). Purified oligonucleotide products were greater than 90% full-length material as determined by polyacrylamide gel electrophoresis analysis.

In Vitro Stability Studies—Oligonucleotides were purified by polyacrylamide gel electrophoresis and desalted using Poly Pak cartridges (Glen Research, Sterling, VA). Labeling was carried out using [γ^{32}P]ATP and T4 polynucleotide kinase. After the labeling reaction, the samples were heated at 95 °C for 2 min to inactivate the T4 polynucleotide kinase for snake venom phosphodiesterase assays. Nuclease stability of the oligonucleotides was assayed at 0.1 μM oligonucleotide using 5×10^{-3} units/ml snake venom phosphodiesterase (U.S. Biochemical Corp.) in a buffer of 50 mM Tris-HCl, pH 8.5, 72 mM CaCl, and 14 mM $MgCl_2$ in a final volume of 50 μl. For *Bal*31 nuclease assays, nuclease stabilities of the oligonucleotides were assayed at 0.1 μM oligonucleotide using 2×10^{-3} units/ml *Bal*31 nuclease (Boehringer Mannheim) in a buffer of 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM EDTA (final volume = 100 μl). For both nuclease assays, 5-μl reaction aliquots were removed at the indicated times, added to an equal volume of 80% formamide containing bromphenol blue and xylene cyanol gel tracking dyes, and then heated for 2 min at 95 °C. Aliquots were then stored at −20 °C until analysis by denaturing polyacrylamide electrophoresis. Quantitation was performed on a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Oligonucleotide Treatment of Cells—T24 cells growing in 10-cm plates at a density of 50–75% confluency were used for oligonucleotide treatments and mRNA analysis. Cells were washed one time with phosphate-buffered saline, prewarmed to 37 °C, and Opti-MEM containing 5 μg/ml DOTMA:DOPE solution for 100 nM oligonucleotide treatments or 12.5 μg/ml DOTMA:DOPE solution for 1 μM oligonucleotide treatments was added to each plate (5 ml/plate). Oligonucleotides were added from 200 μM stocks to each plate and incubated 4 h at 37 °C. Following treatment, medium was removed and replaced with prewarmed McCoy's medium containing 10% fetal bovine serum, and the cells were incubated 37 °C.

Northern Blot Analysis—Total RNA was prepared from cells by the guanidinium isothiocyanate procedure (33) 24–72 h (as indicated under "Results") following initiation of oligonucleotide treatment. Total RNA was isolated by centrifugation of the cell lysates over a cesium chloride cushion (33). RNA samples were electrophoresed through 1.2% agarose-formaldehyde gels and transferred to Zeta-Probe hybridization membranes (Bio-Rad) by capillary diffusion over a 12–14-h period. The RNA was cross-linked to the membrane by exposure to ultraviolet light in a Stratalinker (Stratagene) and hybridized to random-primed ^{32}P-labeled full-length cDNA probes corresponding to human Ha-*ras* or human glyceraldehyde-3-phosphate dehydrogenase. RNA was quantitated using a Molecular Dynamics PhosphorImager as described previously (34).

RESULTS

Phosphorothioate/Phosphodiester Chimeras—In previous reports, oligonucleotide phosphorothioates (P=S) of varying lengths were tested for antisense activity and selectivity for Ha-*ras* mRNA containing a G → T transversion at codon 12 (27, 28). In those reports, antisense activity was shown to correlate directly with relative affinity of an oligonucleotide for its RNA target and on the ability of the oligonucleotide to activate RNase H cleavage *in vitro* using HeLa cell extracts (22, 23). The oligonucleotide that conferred the greatest mutant selectivity in those reports was a 17-mer, the sequence of which is shown in Fig. 1.

Based on the sequence of the mutant selective 17-mer oligonucleotide, a series of chimeric P=S oligonucleotides were syn-

[1] The abbreviations used are: DOTMA:DOPE, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride; P=S, phosphorothioate; P=O, phosphodiester; oligo, oligonucleotide.

Oligo	Sequence
1	Cs Cs As Cs As Cs Cs Gs As Cs Gs Gs Cs Gs Cs Cs C
2	Cs Cs As Cs As Cs Cs Gs AoCs Gs Gs Cs Gs Cs Cs C
3	Cs Cs As Cs As Cs Cs GoAoCs Gs Gs Cs Gs Cs Cs C
4	Cs Cs As Cs As Cs Cs GoAoCoGs Gs Cs Gs Cs Cs C
5	Cs Cs As Cs As Cs CoGoAoCoGs Gs Cs Gs Cs Cs C
6	Cs Cs As Cs As Cs CoGoAoCoGoGs Cs Gs Cs Cs C
7	Cs Cs As CoAoCoCoGoAoCoGoGoCoGs Cs Cs C
8	CoCoAoCoAoCoCoGoAoCoGoGoCoGoCoCoC
9	CoCs AoCs AoCs CoGs AoCs GoGs CoGs CoCs C

FIG. 1. **Design of phosphorothioate/phosphodiester chimeric antisense oligonucleotides.** The 17-mer antisense oligonucleotide sequence is targeted to the mutated (GGC → GUC) codon 12 region of human Ha-*ras* mRNA expressed in the bladder carcinoma cell line T24 (27, 29, 30). Phosphorothioate backbone linkages are indicated by a *lowercase s* between bases; phosphodiester backbone linkages are indicated by a *lowercase o* between bases and are *underlined*. Oligonucleotide sequence is shown 5′ to 3′.

thesized that contain between 1 and 10 centered phosphodiester (P=O) linkages. These oligonucleotides, along with a nonchimeric P=S 17-mer and a uniform P=O 17-mer, are shown in Fig. 1. These compounds were characterized for endonuclease sensitivity *in vitro* using *Bal*31 endonuclease (Fig. 2).

The uniform P=S and the chimera containing a single P=O linkage (oligos 1 and 2) were both totally resistant to endonuclease degradation. Additionally, nuclease sensitivity correlated directly with the number of P=O linkages ("P=O content") for chimeras containing 2 or more P=O linkages. The greatest increase in nuclease sensitivity occurred when P=O content was increased from three linkages to four (oligos 4 and 5, respectively). Finally, all of the chimeras were less sensitive to nuclease degradation as compared with the uniform P=O oligonucleotide, with the exception of oligo 7 which contains 10 consecutive P=O linkages.

To determine the ability of P=S/P=O chimeric oligonucleotides to elicit antisense effects in cells, Ha-*ras* transformed T24 cells were treated in culture with oligonucleotides 1–8 (Fig. 1) at a final oligonucleotide concentration of either 0.1 μM or 1.0 μM and antisense activity was assessed by analysis of Ha-*ras* mRNA expression. As shown in Fig. 3, oligonucleotide-mediated inhibition of Ha-*ras* mRNA expression was dose-dependent. At the high dose (1.0 μM), complete inhibition of Ha-*ras* mRNA expression was observed for oligonucleotides 1–6 (Fig. 3*C*). However, at the low dose (0.1 μM), the relative antisense activity between oligonucleotides containing different amounts of P=O linkages could be readily distinguished. Chimeras containing one or two P=O linkages displayed activity equal to that of the parent uniform P=S. However, as P=O content was increased beyond two linkages, antisense activity gradually decreased. Antisense activity was not observed at either dose for the chimera containing 10 consecutive P=O linkages (oligo 7) nor with the uniform phosphodiester (oligo 8).

The kinetics of antisense inhibition of Ha-*ras* mRNA was also determined for the P= S/P=O chimeric oligonucleotide series (Fig. 4). In this analysis, T24 cells were treated with a relatively low concentration of antisense oligonucleotide (0.1 μM) and Ha-*ras* mRNA levels were determined at 4, 10, and 24 h following initiation of oligonucleotide treatment. The degree of antisense activity induced by the chimeric oligonucleotides was found to be highly time-dependent (Fig. 4). Oligonucleotides containing 0, 1, or 2 P=O linkages all displayed the greatest activity, which was equal and maintained throughout the analysis (up to 24 h). Oligonucleotides containing 3–5 consecutive P=O linkages displayed good to moderate activity at the early time point (4 h) but substantially diminished activity over time. This loss in activity over time for oligonucleotides 4–6 correlated well with the loss of intact oligonucleotide over time in these cells as determined by capillary gel electrophoresis.[2] Oligonucleotide 7, which contains 10 consecutive P=O linkages, as well as oligonucleoride 10, which contains eight alternating P=O linkages, displayed no significant antisense activity at any time following oligonucleotide administration.

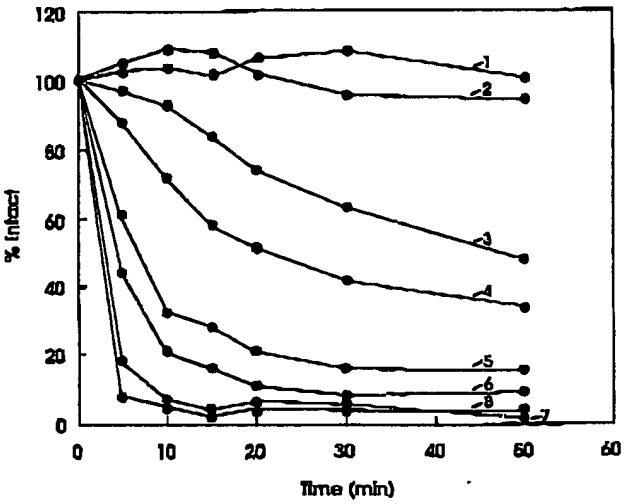

FIG. 2. ***Bal*31 endonuclease degradation of phosphorothioate/phosphodiester chimeric oligonucleotides.** Oligonucleotides 1–8 (Fig. 1) were incubated with *Bal*31 nuclease for the indicated times as described under "Materials and Methods." Full-length ("intact") and digested oligonucleotides were resolved on 20% denaturing polyacrylamide gels, and quantitation of full-length (17-mer) oligonucleotide was performed as described under "Materials and Methods." The *number* next to each digestion curve refers to the oligonucleotide numbers described in Fig. 1. Percentage intact was calculated by comparison with full-length oligonucleotide levels in samples that did not receive *Bal*31 nuclease. The results are representative of three independent experiments.

2′-Sugar-modified Chimeras—A series of 2′-alkoxy and 2′-fluoro, sugar-modified oligonucleotides were analyzed for their ability to confer both resistance to snake venom phosphodiesterase exonuclease activity *in vitro* and antisense activity

[2] B. P. Monia, unpublished experiments.

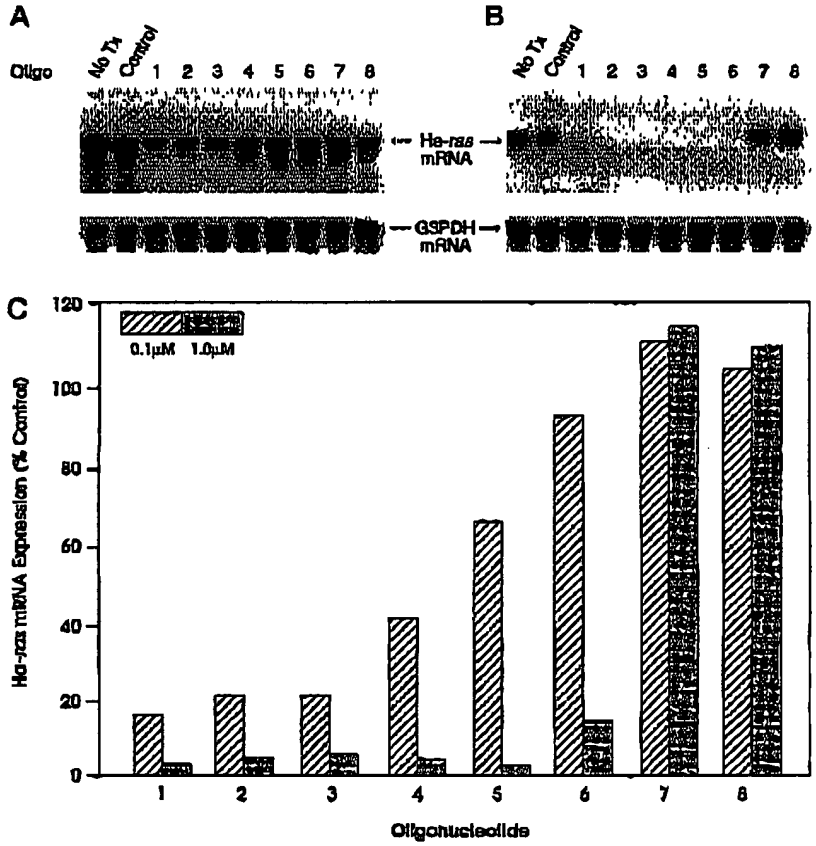

FIG. 3. **Inhibition of Ha-*ras* mRNA expression by phosphorothioate/phosphodiester chimeric antisense oligonucleotides in cultured T24 cells.** Ha-*ras* and glyceraldehyde-3-phosphate dehydrogenase mRNA levels were determined by Northern blot techniques in T24 cells treated with antisense oligonucleotides at a concentration of either 0.1 μM (*A*) or 1.0 μM (*B*). *No Tx* refers to no oligonucleotide treatment; *Control* refers to treatment with a mismatch control uniform phosphorothioate oligonucleotide (CCGCACCGTCGGAGCCC); *numbers 1–8* refer to treatment of cells with oligonucleotides 1–8, which are described in Fig. 1. *C*, quantitation of normalized Ha-*ras* mRNA levels shown in *panels A* and *B*. Quantitation was performed by PhosphorImager analysis, as described under "Materials and Methods." Percentage of control was calculated by comparison with Ha-*ras* mRNA levels in cells that did not receive oligonucleotide. The results are representative of two independent experiments.

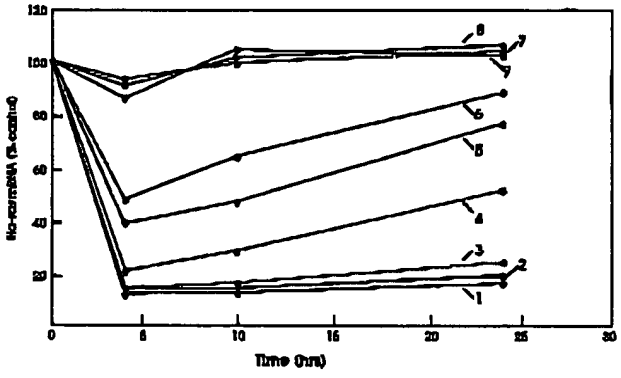

FIG. 4. **Kinetic analysis of Ha-*ras* mRNA reduction in cultured T24 cells by phosphorothioate/phosphodiester chimeric antisense oligonucleotides.** T24 cells were treated with 0.1 μM of the indicated oligonucleotide (oligonucleotides 1–9 as described in Fig. 1), and Ha-*ras* mRNA levels were determined at the indicated times following initiation of oligonucleotide treatment, as described under "Materials and Methods." Percentage of control was calculated as described in Fig. 3. The results are representative of two independent experiments.

against Ha-*ras* in intact cells. Since these modifications have been reported to be unable to support RNase H cleavage *in vitro* (28), and since three or more consecutive deoxy/P=O linkages greatly compromises the antisense activity observed in cells (Figs. 2–4), the Ha-*ras* mutant-selective 17-mer test sequence was designed as a chimera containing 2'-sugar-modified/P=O regions flanking a centered deoxy/P=S "gap" that is of sufficient length to support RNase H activity *in vitro* and antisense activity in cells (28). In addition, oligonucleotides of identical design but containing a uniform P=S backbone were synthesized and tested for comparative antisense activity. The sugar modifications included in this series were methoxy, propoxy, pentoxy, and fluoro. These modifications have previously been reported to markedly affect affinity for a complementary RNA sequence with a rank order (high to low affinity) fluoro > methoxy > propoxy > pentoxy = deoxy (28). The design of these chimeric oligonucleotides along with the chemical structures of the sugar modifications examined in this study are illustrated in Fig. 5.

Significant exonuclease resistance, relative to the unmodified deoxy/P=O chimera, was observed for all of the 2'-alkoxy-modified oligonucleotides (Fig. 6). However, the degree of resistance conferred by a given 2'-alkoxy modification was dependent on the length of the alkoxy chain. The pentoxy modification clearly conferred the greatest exonuclease resistance, displaying stability to degradation equal to that of the uniform deoxy/phosphorothioate ($t_{1/2}$ > 5 h). The 2'-propoxy chimera displayed resistance dramatically less than that of the pentoxy chimera but slightly better than that displayed by the methoxy chimera (propoxy $t_{1/2}$ = 60 min; methoxy $t_{1/2}$ = 30 min).

Oligonucleotide	Design	2' Substituent/Structure
1	Cs Cs As Cs As Cs Cs Gs As Cs Gs Gs Cs Gs Cs Cs C	Deoxy
10	Co Co Ao Co Ao Cs Cs Gs As Cs Gs Gs Co Go Co Co C	
11	Cs Cs As Cs As Cs Cs Gs As Cs Gs Gs Cs Gs Cs Cs C	Methoxy
12	Co Co Ao Co Ao Cs Cs Gs As Cs Gs Gs Co Go Co Co C	
13	Cs Cs As Cs As Cs Cs Gs As Cs Gs Gs Cs Gs Cs Cs C	Propoxy
14	Co Co Ao Co Ao Cs Cs Gs As Cs Gs Gs Co Go Co Co C	
15	Cs Cs Ao Cs Ao Cs Cs Gs As Cs Gs Gs Cs Go Cs Cs C	Pentoxy
16	Co Co As Co As Cs Cs Gs As Cs Gs Gs Co Go Co Co C	
17	Cs Cs As Cs As Cs Cs Gs As Cs Gs Gs Cs Gs Cs Cs C	Fluoro
18	Co Co Ao Co Ao Cs Cs Gs As Cs Gs Gs Co Go Co Co C	

FIG. 5 **Design of 2'-sugar-modified chimeric antisense oligonucleotides.** The 17-mer antisense oligonucleotide sequence targeted to mutated (GGC → GUC) Ha-*ras* codon 12 mRNA was synthesized with the indicated 2'-sugar modification (*boxed sequence*) flanking a centered, "RNase H-sensitive" deoxyphosphorothioate region of seven nucleotides. The 2'-modified regions were synthesized with either phosphorothioate (*lowercase s* between bases) or phosphodiester (*lowercase o* between bases) backbone linkages. In addition, a uniform deoxyphosphodiester/phosphorothioate chimeric oligonucleotide was synthesized (oligonucleotide 10). Oligonucleotide sequence is shown 5' to 3'.

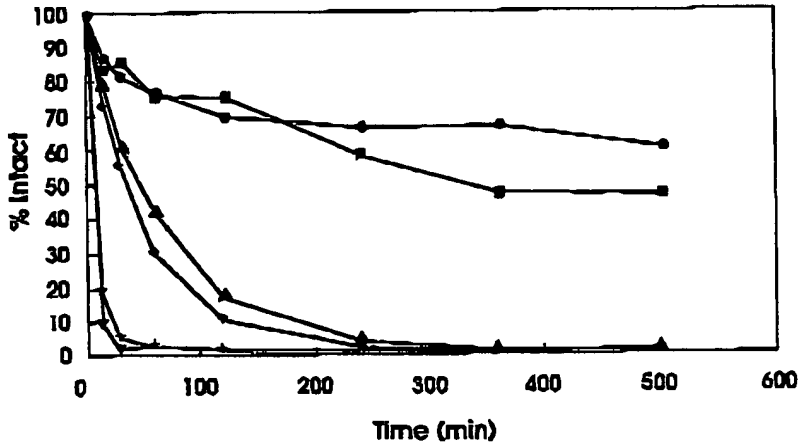

FIG. 6. **Snake venom phosphodiesterase degradation of 2'-sugar-modified chimeric oligonucleotides.** Oligonucleotides were incubated with snake venom phosphodiesterase for the indicated times as described under "Materials and Methods." Full-length ("intact") and digested oligonucleotides were resolved on 20% denaturing polyacrylamide gels, and full-length oligonucleotide was quantitated at the indicated time points as described under "Materials and Methods." Oligonucleotides tested are described in Fig. 5 and are indicated as follows. ■, uniform deoxyphosphorothioate (oligo 1); ●, 2'-pentoxy/P=O chimera (oligo 16); ▲, 2'-propoxy/P=O chimera (oligo 14); ◆, 2'-methoxy/P=O chimera (oligo 12); ▼, 2'-fluoro/P=O chimera (oligo 18); ×, 2'-deoxy/P = O chimera (oligo 10). Percentage intact was calculated as described in Fig. 2. The results are representative of three independent experiments.

The 2'-fluoro-modified chimera displayed no enhanced exonuclease resistance, as compared with the unmodified deoxy/P=O chimera ($t_{1/2}$ < 5 min).

The results described above indicate that the rank order resistance to exonucleolytic degradation conferred by the 2'-sugar modifications tested in this study are pentoxy > propoxy > methoxy > fluoro = deoxy. To determine whether these *in vitro* results correlate with antisense activity in intact cells, these same oligonucleotides were tested for inhibition of Ha-*ras* mRNA expression at two doses (0.1 μM and 1.0 μM). As shown in Fig. 7, antisense activity of the 2'-modified chimeric series was dose-dependent. As previously reported (28), all of the chimeras containing a uniform P=S backbone displayed potent activity at both low and high oligonucleotide concentrations. However, the level of activity achieved for these chimeras differed, depending on the particular modification, with the most potent modification in the uniform P=S backbone being the 2'-fluoro followed by the 2'-methoxy and 2'-propoxy. The 2'-pentoxy chimera displayed activity equal to that of the parent deoxy P=S oligonucleotide. This rank order potency for these 2'-modified chimeras (fluoro > methoxy > propoxy > pentoxy = deoxy) correlates directly with the affinity of these molecules for their complementary RNA sequence (28).

The relative activity of chimeras containing 2'-modifications in a P=O backbone was markedly different from that of the uniform P=S 2'-modified chimeras (Fig. 7). In this case, activity did not correlate with relative affinity for the complementary RNA sequence but instead correlated with the snake venom phosphodiesterase exonuclease resistance conferred by the particular 2'-modification (Fig. 6). The exonuclease-resistant 2'-alkoxy-modified chimeras all displayed dose-dependent activity. However, the most active oligos in this series contained 2'-pentoxy or 2'-propoxy modifications. The 2'-methoxy chimeras displayed intermediate activity, and the 2'-fluoro and unmodified deoxy chimeras displayed no antisense activity at either of the employed concentrations.

The kinetics of antisense inhibition of Ha-*ras* mRNA expression by the 2'-modified P=S/P=O chimeras was also examined

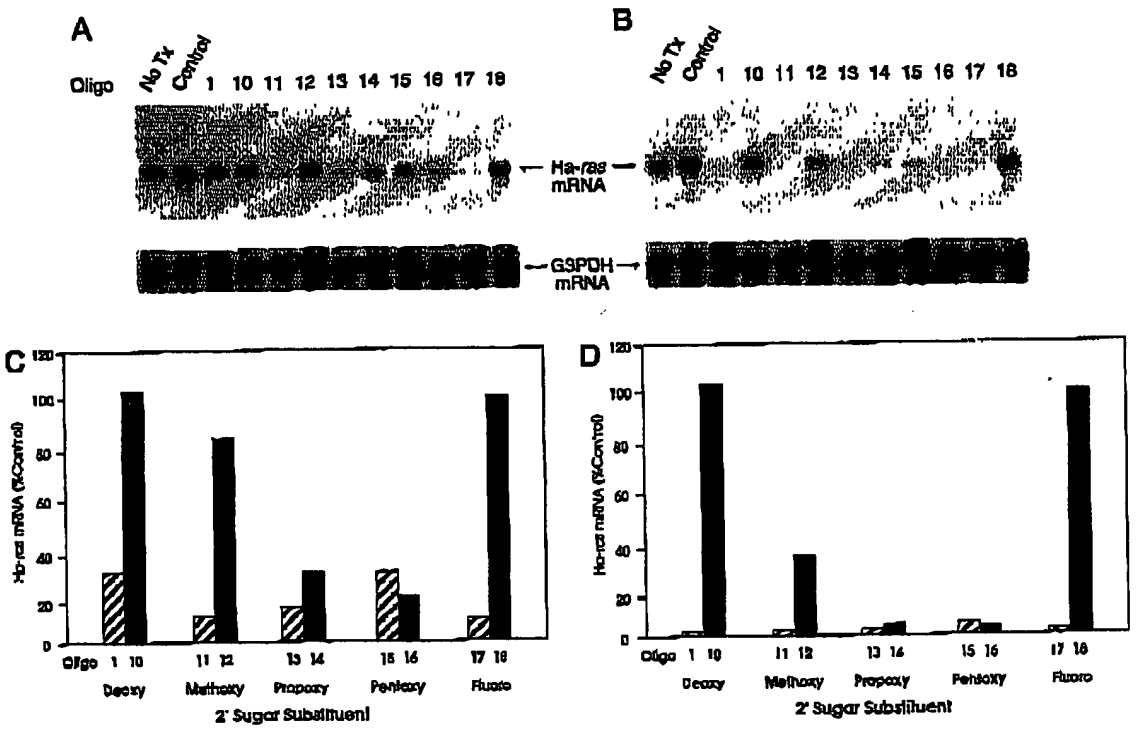

FIG. 7. **Inhibition of Ha-*ras* mRNA expression by 2'-sugar-modified chimeric antisense oligonucleotides in cultured T24 cells.** Ha-*ras* and glyceraldehyde-3-phosphate dehydrogenase mRNA levels were determined by Northern blot techniques in T24 cells treated with modified antisense oligonucleotides at a concentration of either 0.1 μM (*A*) or 1.0 μM (*B*). *No Tx* refers to untreated cells; *Control* refers to treatment with a mismatched control uniform phosphorothioate oligodeoxynucleotide (CCGCACCGTCGGAGCCC); *numbers 1* and *10–18* refer to treatment of cells with oligonucleotides 1 and 10–18, which are described in Fig. 1. *C*, quantitation of normalized Ha-*ras* mRNA levels shown in *panel A* (0.1 μM treatment); *D*, quantitation of normalized Ha-*ras* mRNA levels shown in *panel B* (1.0 μM treatment). For *panels C* and *D*, *hatched bars* indicate 2'-modified/P=O backbones; *solid bars* indicate 2'-modified P=S backbones. Quantitation was performed as described under "Materials and Methods." Percentage of control was calculated as described in Fig. 3. The results are representative of three independent experiments.

in T24 cells treated with low oligonucleotide doses (0.1 μM). (Fig. 8). Duration of antisense activity for a particular 2'-modified oligonucleotide correlated directly with the relative resistance to snake venom phosphodiesterase-mediated exonuclease degradation *in vitro* (Fig. 5). For example, at 4 h following oligonucleotide treatment, the extent of activity for pentoxy, propoxy, and methoxy chimeras was virtually indistinguishable. However, at 48 h following initiation of oligonucleotide treatment, a clear rank order activity was apparent (pentoxy > propoxy > methoxy). At no time point did the 2'-fluoro-modified P=S/P=O chimera or the unmodified (deoxy) P=S/P=O chimera display significant activity. These results demonstrate that, in a P=O backbone, relative activity of a modified oligonucleotide is strongly dependent on the degree of nuclease stability conferred by oligonucleotide modifications regardless of relative affinity for the target mRNA.

DISCUSSION

We have previously identified a 17-mer phosphorothioate antisense oligonucleotide targeted to the Ha-*ras* codon 12 point mutation (GGC → GTC), which displays point mutation specificity for mutated forms of Ha-*ras* (27). We have also shown previously that incorporation of high affinity 2'-sugar modifications within this uniform phosphorothioate oligonucleotide increases antisense potency up to 15-fold in a manner that correlates directly with increased target affinity conferred by the particular 2'-sugar modification (28). In that study, relative nuclease stability of the modified oligonucleotides did not contribute significantly to relative antisense activity, since activity was assessed at short times following oligonucleotide treatment and the oligonucleotides were synthesized as stable uniform phosphorothioates.

In this report, we have used this 17-mer sequence to test the effects of replacing P=S backbone linkages with P=O linkages on both nuclease stability *in vitro* and antisense activity in cells. We also determined the relative stability of oligonucleotides containing various 2'-sugar modifications against nuclease degradation *in vitro* and their relative activity for inhibiting target gene expression in cells. Our results demonstrate a clear correlation between the *in vitro* nuclease stability and antisense activity of the tested modified oligonucleotides. Replacement of P=S linkages with P=O linkages was found to greatly reduce both stability toward nucleases *in vitro* and antisense activity in cells. Furthermore, a direct correlation was observed between the number of P=O linkages introduced into an oligonucleotide ("P=O content") and nuclease sensitivity. However, reduced antisense activity resulting from the introduction of P=O linkages was overcome to some extent through the utilization of higher oligonucleotide concentrations or by examining antisense effects at shorter time periods following the initiation of oligonucleotide treatment.

In agreement with a previous report (24), 2'-alkoxy modifications introduced into a P=O backbone were found to increase stability toward snake venom phosphodiesterase in a manner that correlated directly with 2'-alkoxy chain length. 2'-pentoxy modifications were found to be the most stabilizing sugar modifications examined in this study. This modification, when tested in a P=O backbone, displayed stability toward exonucleolytic cleavage *in vitro* and antisense activity in cells equal to that of a uniform deoxy/P=S oligonucleotide of the same sequence. 2'-propoxy/P=O and 2'-methoxy/P=O oligonucleo-

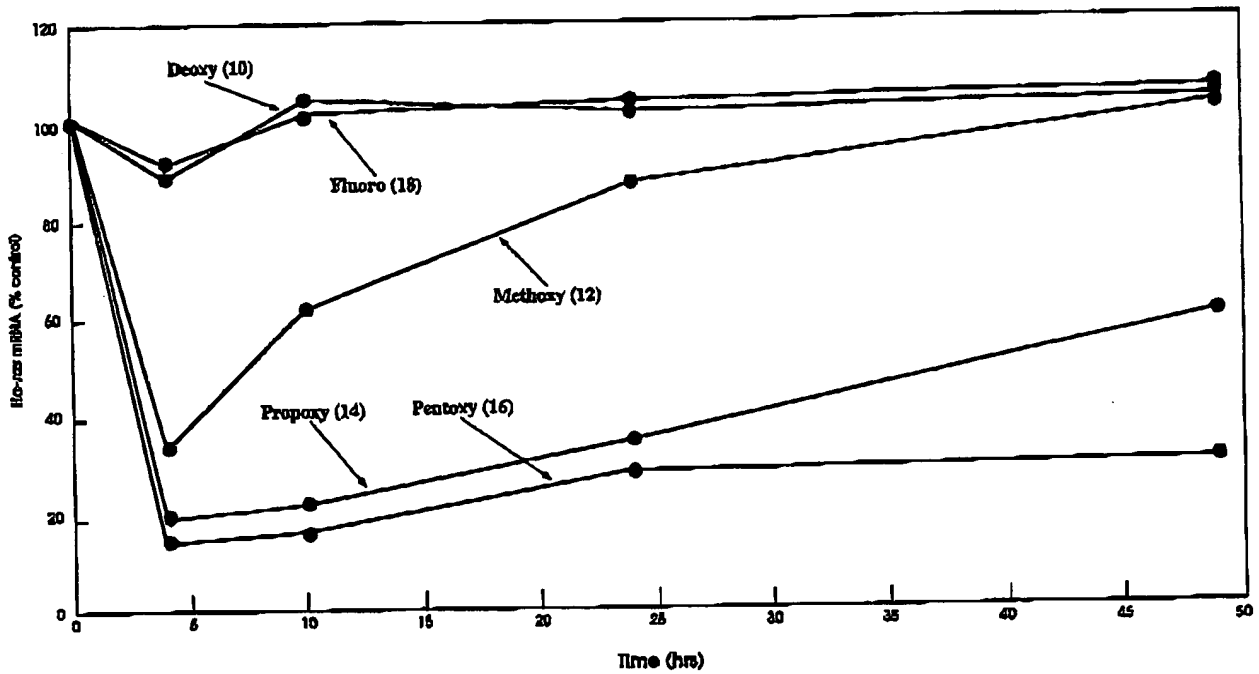

FIG. 8. **Kinetic analysis of Ha-*ras* mRNA reduction in cultured T24 cells by 2'-sugar-modified chimeric antisense oligonucleotides.** T24 cells were treated with 0.1 μM of the indicated oligonucleotide (oligonucleotides 10, 12, 14, 16, and 18, as described in Fig. 5)., and Ha-*ras* mRNA levels were determined at the indicated times following initiation of oligonucleotide treatment, as described under "Materials and Methods." Percentage of control was calculated as described in Fig. 3. The results are representative of two independent experiments.

tides were also found to confer significant nuclease resistance relative to a deoxy/P=O oligonucleotide. As was the case for the 2'-pentoxy/P=O modifications, these 2'-alkoxy P=O modified oligonucleotides also elicited significant antisense effects in cells that were both dose- and time-dependent and in a manner that correlated with relative stability against nucleolytic degradation *in vitro*. A 2'-fluoro/P=O modified oligonucleotide displayed sensitivity toward snake venom phosphodiesterase equal to that of the unmodified deoxyphosphodiester oligonucleotide and was completely ineffective as an antisense agent in cells. These results demonstrate that appropriate 2'-sugar oligonucleotide modifications are attractive alternatives for the design of effective nuclease-stable antisense molecules with reduced phosphorothioate content.

All of the 2'-sugar modifications described above were also tested as chimeras having a uniform P=S backbone for antisense activity in cells. As we have previously reported (28), all of the 2'-sugar-modified P=S chimeras displayed potent antisense activity in cells. However, the level of activity achieved for these chimeras differed, depending on relative affinity for their target RNA and not on relative nuclease resistance (relative affinity = fluoro > methoxy > propoxy > pentoxy = deoxy). Thus, for 2'-modified/P=O oligonucleotides, antisense potency is most dependent on the degree of nuclease stability conferred by the particular 2'-sugar modification. However, for oligonucleotides stabilized by uniform P=S incorporation, additional nuclease sensitivity is not an important determinant of antisense activity when tested up to 48 h following oligonucleotide treatment. Under these conditions, antisense activity is primarily determined by the relative affinity of the oligonucleotide for its target RNA, which is conferred by a particular 2'-sugar modification.

In addition to the studies described above, we have also examined the nuclease sensitivity and antisense activity of 2'-modified chimeras in uniform P=S backbones over very long periods of time following oligonucleotide treatment (>60 h). In these studies, we have observed that combination of nuclease-resistant sugar modifications and nuclease-resistant backbone modifications yields antisense molecules possessing levels of nuclease resistance far greater than that of simple P=S oligonucleotides.[2] These findings suggest that when the level of nuclease resistance required for a particular oligonucleotide application is even greater than that conferred by phosphorothioate oligodeoxynucleotides, combinations of nuclease-resistant backbone modifications with nuclease-resistant sugar modifications may be the design of choice. Such an application has been suggested by Agrawal and colleagues (35, 36), who demonstrated oral bioavailability in rodents with a 2'-methoxy chimeric phosphorothioate. The study reported here suggests that utilization of other 2'-modifications that display even greater nuclease resistance than 2'-methoxy (*e.g.* propoxy and pentoxy), when incorporated into a P=S backbone, may yield oligonucleotides with even greater bioavailability than 2'-methoxy chimeric P=S oligonucleotides. Studies examining these and other pharmacokinetic parameters for 2'-modified antisense oligonucleotides are in progress.

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Properties of Cloned and Expressed Human RNase H1*

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We have characterized cloned His-tag human RNase H1. The activity of the enzyme exhibited a bell-shaped response to divalent cations and pH. The optimum conditions for catalysis consisted of 1 mM Mg^{2+} and pH 7–8. In the presence of Mg^{2+}, Mn^{2+} was inhibitory. Human RNase H1 shares many enzymatic properties with *Escherichia coli* RNase H1. The human enzyme cleaves RNA in a DNA-RNA duplex resulting in products with 5′-phosphate and 3′-hydroxy termini, can cleave overhanging single strand RNA adjacent to a DNA-RNA duplex, and is unable to cleave substrates in which either the RNA or DNA strand has 2′ modifications at the cleavage site. Human RNase H1 binds selectively to "A-form"-type duplexes with approximately 10–20-fold greater affinity than that observed for *E. coli* RNase H1. The human enzyme displays a greater initial rate of cleavage of a heteroduplex-containing RNA-phosphorothioate DNA than an RNA-DNA duplex. Unlike the *E. coli* enzyme, human RNase H1 displays a strong positional preference for cleavage, *i.e.* it cleaves between 8 and 12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex. Within the preferred cleavage site, the enzyme displays modest sequence preference with GU being a preferred dinucleotide. The enzyme is inhibited by single-strand phosphorothioate oligonucleotides and displays no evidence of processivity. The minimum RNA-DNA duplex length that supports cleavage is 6 base pairs, and the minimum RNA-DNA "gap size" that supports cleavage is 5 base pairs.

RNase H1 hydrolyzes RNA in RNA-DNA duplexes (1). Proteins with RNase H activity have been isolated from numerous organisms ranging from viruses to mammalian cells and tissues (2–7). Although RNase H isotypes vary substantially in molecular weight and associated functions, the nuclease properties of the enzymes are similar. All RNase H enzymes, for example, function as endonucleases, specifically cleave RNA in RNA-DNA duplexes, require divalent cations, and generate products with 5′-phosphate and 3′-hydroxyl termini (7).

In prokaryotes, three classes of RNase H enzymes, RNase H1, H2, and H3, have been identified. RNase H2 and H3 share significant sequence homology, whereas RNase H3 and RNase H1 share similar divalent cation preference and cleavage properties. Of the three classes, RNase H2 appears to be the most ubiquitous (8). To date no organism has been shown to express active forms of all three classes of RNase H. The best characterized of the prokaryotic enzymes is *Escherichia coli* RNase H1 (9–13). This enzyme is believed to be involved in DNA replication (14). The key amino acids involved in metal binding, substrate binding, and catalysis have been identified and are highly conserved in the RNase H family (12, 15–17). Furthermore, the enzyme-substrate interaction has been elucidated based on both the three-dimensional structure of the enzyme as well as chemical and structural modification of the heteroduplex substrate (10, 13, 18–21).

RNase H has also been shown to be involved in viral replication. RNase H domains have been identified in viral reverse transcriptases, and these typically share homology with *E. coli* RNase H1 (15). The RNase H portion of the enzyme has been shown to cleave the viral RNA strand producing RNA primers for second strand DNA synthesis, thereby converting the viral RNA into double strand DNA (22).

Two classes of RNase H enzymes have been identified in mammalian cells (2–6). They were reported to differ with respect to co-factor requirements and activity. For example, RNase H type 1 has been shown to be activated by both Mg^{2+} and Mn^{2+} and was active in the presence of sulfhydryl reagents, whereas RNase H type 2 was shown to be activated by only Mg^{2+} and inhibited by Mn^{2+} and sulfhydryl reagents (6). Although the biological roles of the mammalian enzymes are not fully understood, it has been suggested that mammalian RNase H type 1 may be involved in replication and that the type 2 enzyme may be involved in transcription (25, 26).

Recently both human RNase H genes have been cloned and expressed (16, 17, 27). In a previous study we have reported the cloning and expression of a His-tag-labeled RNase H from human cells (16). The human enzyme was homologous to *E. coli* RNase H1. However, its biochemical properties were similar to those reported for the partially purified RNase H type 2. Because it was the first human enzyme to be cloned, it is referred to as human RNase H1. Additionally, a second human RNase H has been cloned (27)[1] but not yet been expressed in an active form. It was shown to be homologous to *E. coli* RNase H2 (28). It is referred to as human RNase H2.

In this communication we provide the first detailed characterization of the enzymological properties of human RNase H1 and compare its properties to those of the homologous protein *E. coli* RNase H1. These studies provide a basis to begin to develop a better understanding of the biological and pharmacological roles of the human RNase H family and to design antisense drugs that interact more effectively with the enzyme.

EXPERIMENTAL PROCEDURES

Materials—T4 polynucleotide kinase was purchased from Promega (Madison, WI). [γ-^{32}P]ATP and [^{32}P]cytidine bisphosphate were purchased from ICN (Irvine, CA). RNase inhibitor was from 5 Prime → 3 Prime, Inc. (Boulder, CO). Calf intestine alkaline phosphatase (CIP)[2] and T4 RNA ligase were purchased from Roche Molecular Biochemicals). Some oligodeoxynucleotides were purchased from Retrogen Inc. (San Diego, CA). The oligodeoxynucleotides were greater than 90% full-length material as determined by capillary gel electrophoresis anal-

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[1] H. Wu, unpublished data.

[2] The abbreviation used is: CIP, calf intestine alkaline phosphatase.

ysis. Human RNase H1 with a His-tag was expressed and purified from a bacterial expression system as described previously (16).

Oligonucleotide Synthesis—Synthesis of 2′-methoxy, 2′-fluoro, 2′-propoxy, and deoxy chimeric oligonucleotides was performed using an Applied Biosystems 380B automated DNA synthesizer as described previously (29, 30). Purification of oligonucleotides was also as described previously (29, 30). Purified oligonucleotides were greater than 90% full-length material as determined by capillary gel electrophoretic analysis.

^{32}P-labeling of RNA Transcripts and Oligoribonucleotides—RNA transcripts and oligoribonucleotides were 5′-end-labeled with ^{32}P using [γ-^{32}P]ATP and T4 polynucleotide kinase (31). Oligoribonucleotides were 3′-end-labeled using [^{32}P]cytidine bisphosphate and T4 RNA ligase. Labeled transcripts and oligonucleotides were purified by electrophoresis on 12% denaturing polyacrylamide gel. The specific activity of the 5′- and 3′-labeled RNAs were, respectively, approximately 6000 and 2000 cpm/fmol.

RNase H Assay Conditions—Hybridization reactions were performed in a variety of reaction buffers (20 mM Tris or NaH_2PO_4 buffer (pH 5.0–10.0), 0–10 mM $MgCl_2$, 0–5 mM $MnCl_2$, 20–120 mM KCl, 0–100 mM NaCl, 0–5 mM *N*-ethylmaleimide, 5% glycerol) containing 100 nM antisense oligonucleotide, 50 nM sense oligoribonucleotide, and 50,000 cpm (per 10-μl reaction volume) ^{32}P-labeled sense oligoribonucleotide. Reactions were heated at 90 °C for 2 min, then cooled, and RNase inhibitor, bovine serum albumin, and 2-mercaptoethanol (final concentration: 1 unit/100 μl, 10 ng/100 μl, and 5 mM, respectively) were added. Samples were equilibrated at 37 °C for at least 4 h and then incubated with human RNase H1. Samples were analyzed using the trichloroacetic acid assay as described previously and polyacrylamide gel electrophoresis (18, 21).

Determination of Initial Rates and Analysis of RNase H Cleavage Sites—Various substrates at different concentrations (10–500 nM RNA, 20–000 nM antisense oligonucleotide) were prepared as described above in the reaction buffer (20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 20 mM KCl, 5% glycerol, 1 unit/100-μl RNase inhibitor, 10 ng/100-μl bovine serum albumin and 5 mM 2-mercaptoethanol). Substrates were incubated with human RNase H1 or *E. coli* RNase H1 and then quenched at specific times. Samples were analyzed by the trichloroacetic acid assay. The amount of substrate hydrolyzed was measured, and the initial rate and Michaelis-Menten parameters (K_m, V_{max}) were calculated (32). Substrate concentrations for trichloroacetic acid assays were the concentrations (nM) of intact duplex in an incubation. The trichloroacetic acid assay compares the amount of 5′ ^{32}P-labeled oligonucleotide that precipitates, thus directly measuring the fraction of duplex that remains intact, and by subtraction, the fraction cleaved to be trichloroacetic acid-soluble. Control studies showed that trichloroacetic acid precipitation was quantitative for single strand oligonucleotides ≥12 nucleotides in length. As the substrates were 5′-labeled, most cleavage products were trichloroacetic acid-soluble. For longer products, the trichloroacetic acid assay may underestimate cleavage; however, polyacrylamide gel electrophoretic analysis confirmed the cleavage rates observed in the trichloroacetic acid assays (data not shown). Consequently, the errors introduced into the trichloroacetic acid assay results by variations in precipitation of oligonucleotides of different lengths must be small. RNase H generated cleavage products were analyzed by a denaturing polyacrylamide gel. A base hydrolysis ladder was prepared by incubation of 5′-end-labeled RNA at 90 °C for 5 min in 100 mM $NaCO_3$ (pH 9.0). The positions of the cleavage sites were determined with oligonucleotide size markers generated by RNases A and T1 (33). The gels were then analyzed and quantified using a Molecular Dynamics PhosphorImager (21).

Determination of Binding Affinity—Binding affinities were determined by competitive inhibition analyses. At various concentrations ($n > 5$) ranging from 10 to 100 nM, the substrates, *i.e.* oligodeoxynucleotide-oligoribonucleotide hybrids, were prepared as described above. The competing substrate analog was prepared in reaction buffer containing equimolar concentrations of the modified sense and antisense oligonucleotides. Following equilibration at 37 °C, the competing substrate analog was added to the wild type substrate reaction, and the mixture was incubated with human RNase H1 in the presence of excess competing substrate, as described above. The samples were analyzed by trichloroacetic acid assay and denaturing polyacrylamide gel analyses. These data were analyzed by both the Lineweaver-Burk and Augustinsson methods to determine if the inhibitors were competitive and to ascertain the inhibitory constants (K_i) for the competing substrates, also as described previously (21, 32, 34).

RESULTS

Properties of Purified Human RNase H1—The effects of various reaction conditions on the activity of human RNase H1 were evaluated (Fig. 1). The optimal pH for the enzyme in both Tris-HCl and phosphate buffers was 7.0–8.0. At pH values above pH 8.0, enzyme activity was reduced. However, this could be due to instability of the substrate or effects on the enzyme, or both. To evaluate the potential contribution of changes in ionic strength to the activities observed at different pH values, two buffers, NaH_2PO_4 and Tris-HCl, were studied at pH 7.0 and gave the same enzyme activity even though the ionic strengths differed. Enzyme activity was inhibited by increasing ionic strength (Fig. 1*B*) and *N*-ethylmaleimide (Fig. 1*C*). Enzyme activity increased as the temperature was raised from 25 to 42 °C (Fig. 1*D*). Mg^{2+} stimulated enzyme activity with an optimal concentration of 1 mM. At higher concentrations, Mg^{2+} was inhibitory (Fig. 1*E*). In the presence of 1 mM Mg^{2+}, Mn^{2+} was inhibitory at all concentrations tested (Fig. 1*F*). The purified enzyme was quite stable and easily handled. In fact, the enzyme could be boiled and rapidly or slowly cooled without significant loss of activity (Fig. 1*D*). The initial rates of cleavage were determined for four duplex substrates studied simultaneously. The initial rate of cleavage for a phosphodiester DNA-RNA duplex was 1050 ± 203 pmol liter^{-1}min^{-1} (Table I*A*). The initial rate of cleavage of a phosphorothioate oligodeoxynucleotide duplex was approximately 4-fold faster than that of the same duplex comprised of a phosphodiester antisense oligodeoxynucleotide (Table I*A*). The initial rates for 17-mer and 20-mer substrates of different sequences were equal (Table I*B*). However, when a 25-mer heteroduplex containing the 17-mer sequence in the center of the duplex was digested (RNA No. 3), the rate was 50% faster. Interestingly, the K_m of the enzyme for the 25-mer duplex was 40% lower than that for the 17-mer, whereas the V_{max} values for both duplexes were the same (see Table III), suggesting that with the increase in length, a larger number of cleavage sites are available, resulting in an increase in the number of productive binding interactions between the enzyme and substrate. As a result, a lower substrate concentration is required for the longer duplex to achieve a cleavage rate equal to that of the shorter duplex.

To better characterize the substrate specificity of human RNase H1, duplexes in which the antisense oligonucleotide was modified in the 2′ position were studied. As previously reported for *E. coli* RNase H1 (18–21), human RNase H1 was unable to cleave substrates with 2′ modifications at the cleavage site of the antisense DNA strand or the sense RNA strand (Table II). For example, the initial rate of cleavage of a duplex containing a phosphorothioate oligodeoxynucleotide and its complement was 3400 pmol liter^{-1}min^{-1}, whereas that of its 2′-propoxy-modified analog was undetectable (Table II). A duplex comprised of a fully modified 2′-methoxy antisense strand also failed to support any cleavage (Table II). The placement of 2′-methoxy modifications around a central region of oligodeoxynucleotides reduced the initial rate (Table II). The smaller the central oligodeoxynucleotide "gap," the lower the initial rate. The smallest "gap-mer" for which cleavage could be measured was a 5 deoxynucleotide gap. These data are highly consistent with observations we have previously reported for *E. coli* RNase H1, except that for the bacterial enzyme, the minimum gap size was 4 deoxynucleotides (18, 20, 21).

The K_m and V_{max} of human RNase H1 for three substrates are shown in Table III. The K_m values for all three substrates were substantially lower than those of *E. coli* RNase H1 (Table III) (18, 19). As previously reported for *E. coli* RNase H1, the K_m for a phosphorothioate-containing duplex was lower than

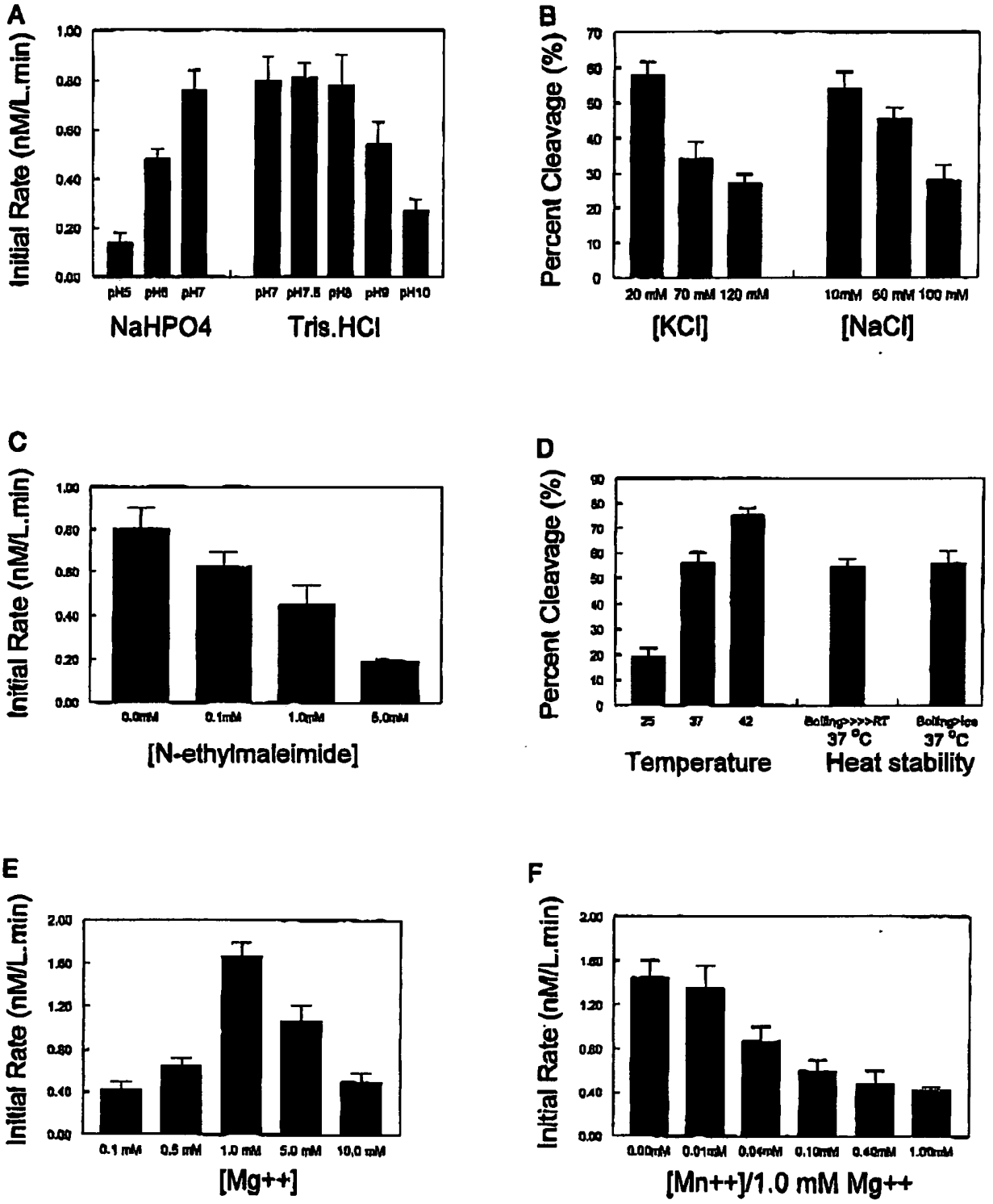

FIG. 1. **Effects of conditions on the human RNase H1 activity.** 5′-End-labeled RNA and antisense oligonucleotides were preannealed and digested with RNase H1 as described under "Experimental Procedures." The final substrate concentration was 20 nM for RNA and 40 nM for antisense oligonucleotide. The activity was measured as either initial rate or percent cleavage. *A*, pH dependence of RNase H activity. The substrate was annealed in phosphate or Tris buffer at different pH values and subjected to RNase H digestion in the presence of 10 mM Mg^{2+}. *B*, effect of ionic strength on RNase H activity. *C*, effect of the sulfhydryl-blocking agent, *N*-ethylmaleimide, on RNase H activity. The substrate was prepared in the same buffer as above without β-mercaptoethanol. *D*, temperature sensitivity and heat stability of the human RNase H1. Enzyme digestion was carried out under different temperatures. Alternatively, the enzyme was boiled for 5 min in buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 20 mM dithiothreitol, and 50% glycerol, then either slowly cooled down to room temperature (*RT*) or rapidly moved into ice bath. *E*, effect of Mg^{2+} on RNase H activity. The substrate was prepared in the same buffer as above with a different concentration of Mg^{2+} and subject to RNase H digestion. *F*, effect of Mn^{2+} on RNase H1 activity. The substrate was digested in the buffer containing 1 mM Mg^{2+} and different concentrations of Mn^{2+}.

that of a phosphodiester duplex. The V_{max} of the human enzyme was 30-fold lower than that of the *E. coli* enzyme. The V_{max} for the phosphorothioate-containing substrate was less than the phosphodiester duplex. This is probably due to inhibition of the enzyme at higher concentrations by excess phosphorothioate single strand oligonucleotide (see below) as the

TABLE I

Effects of phosphorothioate substitution and substrate length on digestion by human RNase H1

Oligoribonucleotides were preannealed with the complementary antisense oligodeoxynucleotide at 10 and 20 nM and subjected to digestion by human RNase H1. The 17-mer (RNA No. 1) and 25-mer (RNA No. 3) RNA sequences are derived from Ha-Ras oncogen (51), and the 20-mer RNA contains the 17-mer sequence. The 20-mer (RNA No. 2) sequence is derived from human hepatitis C virus core protein coding sequence (52). The initial rates were determined as described under "Experimental Procedures." A, comparison of the initial rates of cleavage of an RNA-phosphodiester (P=O) and an RNA-phosphorothioate (P=S) duplexes. B, comparison among duplexes of different sequences and lengths.

RNA No.	RNA	Antisense DNA	Initial rate
			pmol liter^{-1} min^{-1}
A			
1	GGGCGCCGUCGGUGUGG	17-mer P=O	1050 ± 203
1	GGGCGCCGUCGGUGUGG	17-mer P=S	4034 ± 266
B			
1	GGGCGCCGUCGGUGUGG	17-mer P=O	1050 ± 203
2	ACUCCACCAUAGUACACUCC	20-mer P=O	1015 ± 264
3	UGGUGGGCGCCGUCGGUGUGGGCAA	25-mer P=O	1502 ± 182

TABLE II

Effects of 2′-substitution and deoxy-gap size on digestion rates by human RNase H1

Substrate duplexes were hybridized, and initial rates were determined as shown in Table 1 and described under "Experimental Procedures." The 17-mer RNA is the same used in Table 1, and the 20-mer RNA (UGGUGGGCAAUGGGCGUGUU, RNA No. 4) was derived from the protein kinase C ζ (53) sequence. The 17-mer and 20-mer P=S oligonucleotides were full deoxyphosphorothioate-containing No. 2′-modifications. The 9, 7, 5, 4, and 3 deoxy gap oligonucleotides were 17-mer oligonucleotide with a central portion consisting of nine, seven, and five, and four deoxynucleotides flanked on both sides by 2′-methoxynucleotides (also see Fig. 2). Boldface sequences indicate the position of the 2′-methoxyl-modified residues. The italic sequences indicates the position of the 2′-propoxy-modified residues.

RNA No.	RNA	Antisense DNA	Initial Rate
			pmol liter^{-1} min^{-1}
1	17-mer	CCACACCGACGGCGCCC	4034 ± 266
	17-mer	CCACACCGACGGCGCCC	1081 ± 168
	17-mer	CCACACCGACGGCGCCC	605 ± 81
	17-mer	CCACACCGACGGCGCCC	830 ± 56
	17-mer	CCACACCGACGGCGCCC	0
	17-mer	CCACACCGACGGCGCCC	0
	17-mer	CCACACCGACGGCGCCC	0
4*	20-mer	AACACGCCCATTGCCCACCA	3400 ± 384
	20-mer	AACACGCCCATTGCCCACCA	0

TABLE III

Kinetic constants for RNase H1 cleavage of RNA-DNA duplexes

The RNA-DNA duplexes in Table I were used to determine K_m and V_{max} of human and *E. coli* RNase H1 as described under "Experimental Procedures."

Substrates	Human RNase H		*E. coli* RNase H1	
	K_m		K_m	V_{max}
	nM	*nmol liter^{-1} min^{-1}*	*nM*	*nmol liter^{-1} min^{-1}*
25-mer Ras (RNA No. 3)-DNA (P=O)	35.4	1.907		
17-mer Ras (RNA No. 1)-DNA (P=O)	56.1	1.961	385	38.8
17-mer Ras (RNA No. 1)-DNA (P=S)	13.9	1.077		

initial rate of cleavage for a phosphorothioate-containing duplex was, in fact, greater than the phosphodiester (Table I).

Binding Affinity and Specificity—To evaluate the binding affinity of human RNase H1, a competitive cleavage assay in which increasing concentrations of noncleavable substrates were added was used (21). Using this approach, the K_i is formally equivalent to the K_d for the competing substrates. Of the noncleavable substrates studied, Lineweaver-Burk analyses demonstrated that all inhibitors shown in Table IV were competitive (data not shown). A duplex containing a phosphodiester oligodeoxynucleotide hybridized to a phosphodiester 2′-methoxy oligonucleotide as the noncleavable substrate is considered most like DNA-RNA. Table IV shows the results of these studies and compares them to previously reported results for the *E. coli* enzyme performed under similar conditions (20, 21). Clearly, the affinity of the human enzyme for its DNA-RNA like substrate (DNA-2′-methoxy) was substantially greater than that of the *E. coli* enzyme, consistent with the differences observed in K_m (Table III).

TABLE IV

Binding constants and specificity of RNase H's

K_d values were determined as described under "Experimental Procedures." The K_d values for *E. coli* RNase H1 were derived from previously reported data (21). The competing substrates (competitive inhibitors) used in the binding study are divided into two categories: single strand (ss) oligonucleotides and oligonucleotide duplexes all with the 17-mer sequence as in Table 1 (RNA No. 1). The single strand oligonucleotides included ssRNA, ssDNA, ss fully modified 2′-methoxy phosphodiester oligonucleotide (ss 2′-methoxy), and ss full phosphorothioate deoxyoligonucleotide (ssDNA, P=S). The duplex substrates include DNA-DNA duplex, RNA-RNA duplex, DNA-fully modified 2′-fluoro or fully modified 2′-methoxy oligonucleotide (DNA-2′-fluoro or 2′-methoxy), RNA-2′-fluoro, or 2′-methoxy duplex. Dissociation constants are derived from ≥3 slopes of Lineweaver-Burk and/or Augustisson analysis. Estimated errors for the dissociation constants are ≤ 2-fold. Specificity is defined by dividing the K_d for a duplex by the K_d for an RNA-RNA duplex.

Inhibitors	Human RNase H1		*E.coli* RNase H1	
	K_d	Specificity	K_d	Specificity
	nM		*nM*	
DNA-2′-methoxy	458	5.8	3400	2.1
RNA-2′-methoxy	409	5.2	3100	1.9
RNA-RNA	79	1.0	1600	1.0
RNA-2′-fluoro	76	1.0		
DNA-2′-fluoro	99	1.3		
DNA-DNA	3608	45.7	176,000	110.0
ssRNA	1400	17.7		
ssDNA	1506	19.6	942,000	588.8
ss2′-methoxy	2304	29.2	118,000	73.8
ssDNA, P=S	36	0.5	14,000	8.8

E. coli RNase H1 displays approximately equal affinity for RNA-RNA, RNA-2′-methoxy, and DNA-2′-methoxy duplexes (Table IV). The human enzyme displays similar binding properties but is more able to discriminate between various duplexes. For example, the K_d for RNA-RNA was approximately 5-fold lower than the K_d for DNA-2′-methoxy. This is further demonstrated by the K_d for the RNA-2′-fluoro duplex. The K_d for the DNA-2′-fluoro duplex was slightly greater than for the RNA-2′-fluoro duplex and the RNA-RNA duplex but clearly lower than for other duplexes. Thus, both enzymes can be considered double strand RNA-binding proteins. However, human RNase H1 is somewhat less specific for duplexes as compared with single strand oligonucleotides than the *E. coli* enzyme. The enzyme bound to single strand RNA and DNA only

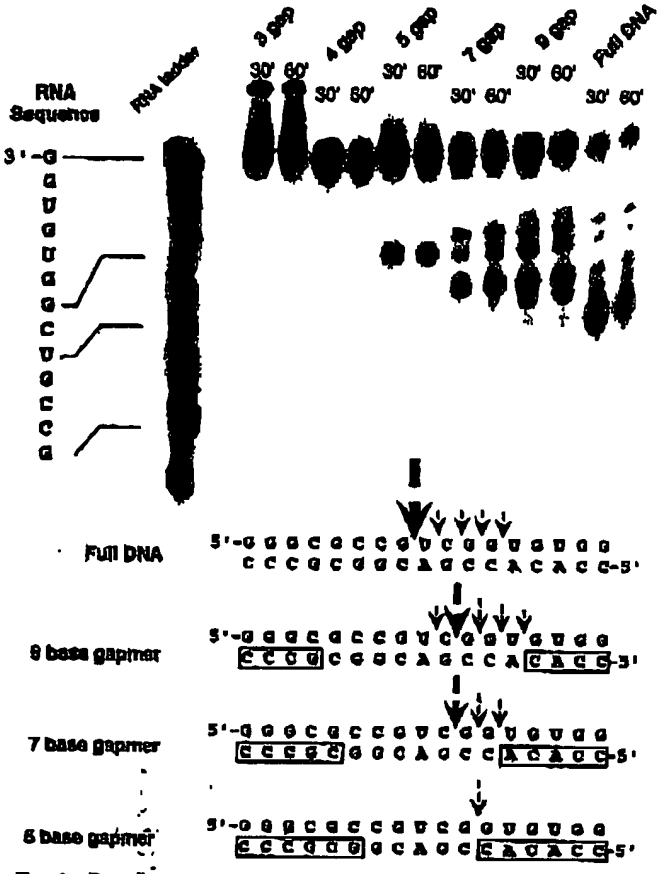

FIG. 2. **Denaturing polyacrylamide gel analysis of human RNase H1 cleavage of 17-mer RNA-DNA gap-mer duplex.** Antisense oligonucleotides were hybridized with 5'-end-labeled sense RNA as described under "Experimental Procedures," then digested with RNase H1 for 30 and 60 min at 37 °C. A base hydrolysis RNA ladder was prepared as described under "Experimental Procedures." The RNA ladder was sequenced with RNases T1, CL3, and A1 (data not shown). For each substrate, the RNA sequences (5′ → 3′) are shown above the DNA sequence. *Boxed sequences* indicate the position of the 2′-methoxy-modified residues. The *arrows* indicate the sites of the enzyme digestion, and the size of the *arrows* reflect the relative cleavage intensities.

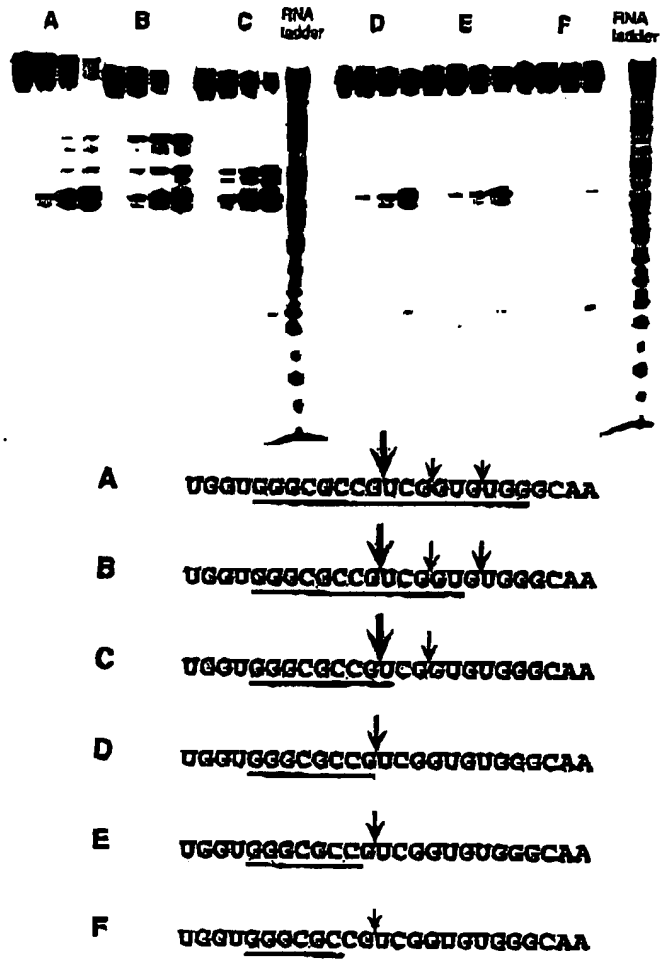

FIG. 3. **Analysis of human RNase H1 cleavage of a 25-mer Ras RNA hybridized with phosphodiester oligodeoxynucleotides of different lengths.** Antisense oligonucleotides with different lengths from 6- to 17-mer were hybridized with 5′-end-labeled 25-mer sense Ha-Ras RNA as described under "Experimental Procedures," then digested with RNase H1 at 37 °C for a time course of 0, 2, 5, and 10 min shown on the gel (left to right) for each substrate (*A* to *F*). A 25-mer RNA ladder was prepared and sequenced as described the legend for Fig. 2. For each substrate, the RNA sequences (5′ → 3′) are shown in the figure, and antisense DNA sequences were indicated by the *solid line* below the RNA sequence. The *arrows* indicate the sites of the enzyme digestion, and the size of *arrows* reflect the relative cleavage intensities.

20-fold less well than an RNA-RNA duplex, whereas the *E. coli* enzyme bound to single strand DNA nearly 600-fold less than to an RNA-RNA duplex (Table IV). The affinity of a single strand phosphorothioate oligodeoxynucleotide for both enzymes was significant relative to the affinity for the natural substrate and accounts for the inhibition of the enzymes by members of this class oligonucleotides. Remarkably, human RNase H1 displayed the highest affinity for a single strand phosphorothioate oligodeoxynucleotide. Thus, this noncleavable substrate is a very effective inhibitor of the enzyme, and excess phosphorothioate antisense drug in cells might be highly inhibitory.

Site and Sequence Preferences for Cleavage—Fig. 2 shows the cleavage pattern for RNA duplexed with its phosphorothioate oligodeoxynucleotide and the pattern for several gap-mers. In the parent duplex, RNA cleavage occurred at a single major site with minor cleavage noted at several sites 3′ to this major cleavage site that was 8 nucleotides from 5′ terminus of the RNA. Note that the preferred site occurred at a GU dinucleotide. Cleavage of several gap-mers occurred more slowly, and the major cleavage site was at a different position from that of the parent duplex. Furthermore, in contrast to the observations we have made for *E. coli* RNase H1 (18), the major cleavage site in gap-mers treated with human RNase H1 did not occur at the nucleotide apposed to the nucleotide adjacent to the first 2′-methoxy nucleotide in the wing hybridized to the 3′ portion of the RNA.

To further evaluate the site and sequence specificities of human RNase H1, cleavage of substrates shown in Figs. 3 and Fig. 4 was studied. In Fig. 3, the sequence of the RNA is displayed below the sequencing gels, and the length and position of the complementary phosphodiester oligodeoxynucleotide is indicated by the *solid line* below the RNA sequence. This figure demonstrates several important properties of the enzyme. First, the main cleavage site was consistently observed 8–9 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex irrespective of whether there were 5′ or 3′-RNA single strand overhangs. Second, the enzyme, like *E. coli* RNase H1 (20, 21), was capable of cleaving single strand regions of RNA adjacent to the 3′ terminus of an RNA-DNA duplex. Third, the minimum duplex length that supported any cleavage was approximately 6 nucleotides. RNase protection assays were used to confirm that under conditions of the assay, the shorter duplexes were fully hybridized, so the differences observed were not due to the failure to hybridize. To assure

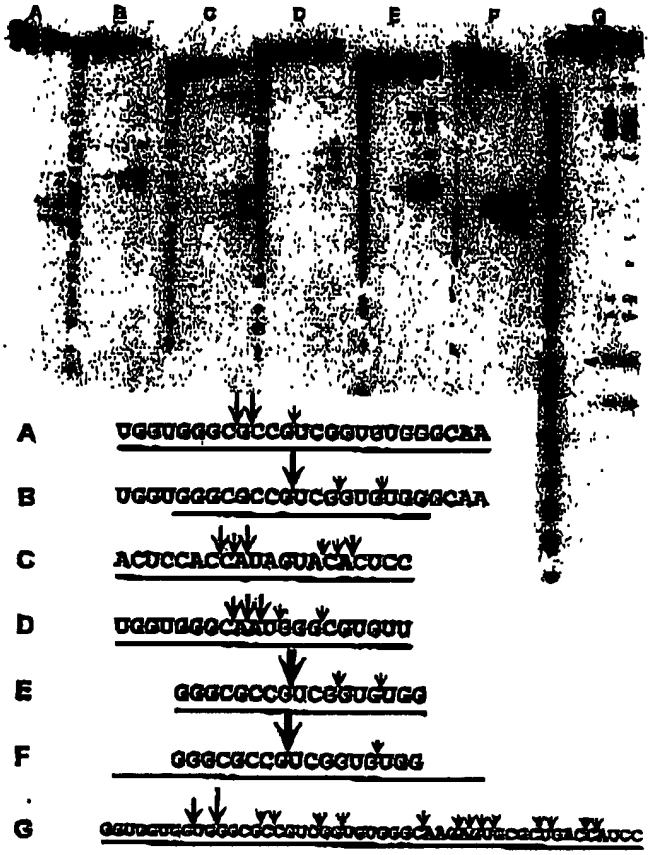

FIG. 4. Analysis of human RNase H1 cleavage of RNA-DNA duplexes with different sequences, length, and 3′ or 5′ overhangs. Antisense oligonucleotides of different sequences and lengths were hybridized with their complementary 5′-end-labeled RNA as described under "Experimental Procedures" and then digested with RNase H1 at 37 °C for 0, 2, 5, or 10 min as shown on the gel (left to right) for each substrate (*A* to *G*). Substrate *A* (25-mer), *B* (25-mer), *E* (17-mer), *F* (17-mer), *G* (47-mer) sequences are from the Harvey-RAS oncogene (51), substrate *C* (20-mer) is from hepatitis C virus (24), and substrate D (20-mer) is from protein kinase C ζ (23). The RNA ladder was prepared and sequenced as described in the legend for Fig. 2. For each substrate, the RNA sequences (5′ → 3′) are shown in the figure, and antisense DNA sequences were represented by the *solid line* below the RNA sequence. The *arrows* indicate the major sites and relative intensities of the enzyme digestion.

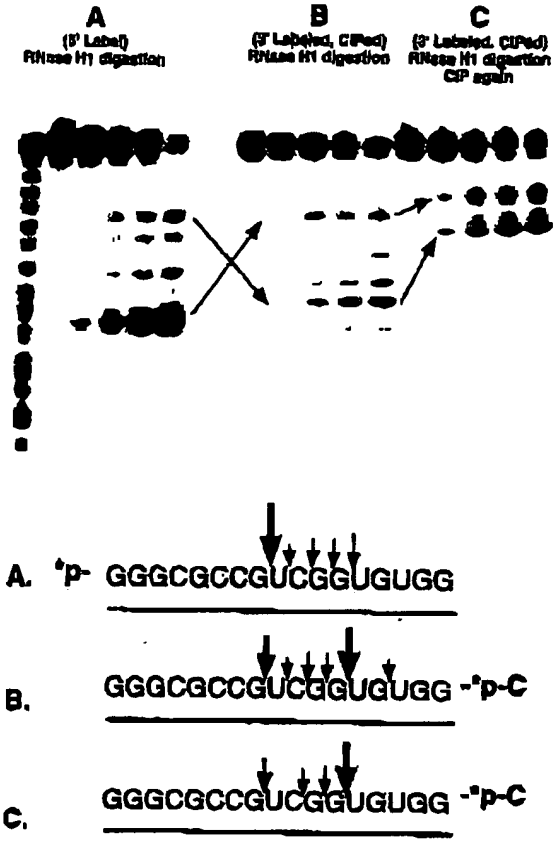

FIG. 5. Product and processivity analysis of human RNase H1 cleavage on 17-mer Ras RNA-DNA duplexes. RNA was either 5′-end-labeled (for *reaction A*) using [γ-^{32}P]ATP and T4 nucleotide kinase or 3′-end-labeled (for *reactions B* and *C*) using [^{32}P]cytidine bisphosphate and T4 RNA ligase as described under "Experimental Procedures." The 3′-end-labeled RNA was further dephosphorylated with calf intestine alkaline phosphatase (*CIPed*) (CIPed: dephosphorylated with CIP). Hybridization reactions were prepared as described in Fig. 1. The digestion with RNase H1 was performed at 37 °C for 0, 2, 5, 10, or 20 min as shown on the gel (left to right) for each substrate (*A* to *C*). Reactions with 3′-labeled substrate were divided into equal aliquots, with 1 aliquot subjected to further dephosphorylation with CIP. The *p- indicates the position of the ^{32}P label. 5′- and 3′-end-labeled duplexes treated with human RNase H1 are shown in *panels A* and *B*, respectively. The 3′-end-labeled hybrid and degradation products treated with CIP after digestion with RNase H1 exhibited slower migration on the polyacrylamide gel due to the loss of the 5′-phosphate (*reaction C*) on the cleavage products. However, as the intact duplex had had its terminal phosphate removed by the previous CIP treatment (*panel C*), its migration was unchanged.

that the 6-nucleotide duplex was fully hybridized, the reactions were carried out at a 50:1 DNA-RNA ratio (data not shown). Fourth, the figure shows that for duplexes smaller than the nine base pairs, the smaller the duplex, the slower the cleavage rate. Fifth, the preferred cleavage site was located at a GU dinucleotide.

The site and sequence specificities are further explored in Fig. 4. That the enzyme displays little sequence preference is demonstrated by comparing the rates and sites of cleavage for duplexes A, C, and D. In all cases, the preferred site of cleavage was 8–12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex irrespective of the sequence. Comparison of the cleavage pattern for duplexes A and B shows that cleavage occurred at the 8–12 nucleotide position even when there were RNA overhangs also as shown in Fig. 3. Cleavage of duplex F demonstrated that the site of cleavage was retained even if there were 5′- and 3′-DNA overhangs. In a longer substrate, duplex G, the main site of cleavage was still 8–12 nucleotides from the terminus of the duplex. However, minor cleavage sites were observed throughout the RNA, suggesting that this substrate might support binding of more than one enzyme molecule/substrate, but that the preferred site was near the 5′-RNA-3′-DNA terminus. Finally, optimal cleavage seemed to occur when a GU dinucleotide was located 8–12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex.

To address both the mechanism of cleavage and processivity, the cleavage of 5′-labeled and 3′-labeled substrates was compared (Fig. 5). *Lane C* shows that CIP treatment before and after digestion with human RNase H1 resulted in a shift in the mobility of the digested fragments, suggesting that human RNase H1 generates cleavage products with 5′-phosphates. Thus, it is similar to *E. coli* RNase H1 in this regard (20). A second intriguing observation is that the addition of [^{32}P]cytidine to the 3′-end of the RNA caused a shift in the position of the preferred cleavage site (*A versus B* or *C*). The four cleavage sites in the center of the duplex observed with a 5′-phosphate-labeled RNA were observed in 3′-[^{32}P]cytidine-labeled substrates. However, the main cleavage site shifted from base pair 8 to base pair 12. Interestingly, the sequence at both sites was GU. Thus, it is conceivable that the enzyme selects a position 8–12 nucleotide from the 5′-RNA-3′-DNA terminus then

cleaves at a preferred dinucleotide such as GU. Third, this figure considered along with the cleavage patterns shown in Figs. 3 and 4 demonstrates that this enzyme displays minimal processivity in either the 5′ or 3′ direction. In no time-course experiment using any substrate have we observed a pattern that would be consistent with processivity. The possibility that the failure to observe processivity in Figs. 3 and 4 was due to processivity in the 3′ to 5′ direction is excluded by the results in Fig. 5. Again, this is significantly different from observations we have previously reported for *E. coli* RNase H1 (18).

DISCUSSION

General Properties of Human RNase H1 Activity—In this study, we have characterized the properties of human RNase H1. As the protein studied is a His-tag fusion and was denatured and refolded, it is possible that the activity of the enzyme in its native state might be greater than we have observed. However, basic properties reported in this paper are certainly likely to reflect the basic properties of the native enzyme. Numerous studies have shown that a His-tag does not interfere with protein folding and crystallization (35, 36), kinetic and catalytic properties (37, 38), or nucleic acid binding properties (39, 40),[2] since it is very small (few amino acids), and its pK is near neutral. As shown in this and our previous (16) studies, this His-tag fusion protein did behave like other RNase H proteins (6, 7). It cleaved specifically the RNA strand in RNA-DNA duplexes, resulted in cleavage products with 5′-phosphate termini (Fig. 5), and was affected by divalent cations (Fig. 1). Optimal conditions for human RNase H1 were similar but not identical to *E. coli* RNase H1. For the human enzyme, the Mg^{2+} optimum was 1 mM, and 5 mM Mg^{2+} was inhibitory. In the presence of Mg^{2+}, both enzymes were inhibited by Mn^{2+}. The human enzyme was inhibited by *N*-ethylmaleimide and was quite stable, easily handled, and did not form multimeric structures (Fig. 1). The ease of handling, denaturation, refolding, and stability in various conditions suggest that the human RNase H1 was active as a monomer and has a relatively stable preferred conformation.

Studies on the structure and enzymatic activities of a number of mutants of *E. coli* RNase H1 have recently led to a hypothesis to explain the effects of divalent cations termed an activation/attenuation model (41). The effects of divalent cations on human RNase H1 are complex and are consistent with the suggested activation/attenuation model. The amino acids proposed to be involved in both cation binding sites are conserved in human RNase H1 (16).

Positional and Sequence Preferences and Processivity—The site and sequence specificity of human RNase H1 differ substantially from *E. coli* RNase H1. Although neither enzyme displays significant sequence specificity (Ref. 18 and Figs. 2–5), the human enzyme displays remarkable site specificity. Figs. 2–4 show that human RNase H1 preferentially cleaved 8–12 nucleotides 3′ from the 5′-RNA-3′-DNA terminus of a DNA-RNA duplex irrespective of whether there were 5′ or 3′-RNA or DNA overhangs. The process by which a position is selected and then within that position on the duplex a particular dinucleotide is cleaved preferentially must be relatively complex and influenced by sequence. Clearly, the dinucleotide, GU, is a preferred sequence. In Fig. 3, for example, all the duplexes contained a GU sequence near the optimal position for the enzyme, and in all cases, the preferential cleavage site was GU. Additionally, in duplexes A and B a second GU was also cleaved, albeit at a very slow rate. The third site in duplexes A and B cleaved was a GG dinucleotide 7 base pairs from the 3′-RNA-5′-DNA terminus. Thus, the data suggest that the enzyme displays strong positional preference and, within the appropriate site, slight preference for GU dinucleotides.

The strong positional preference exhibited by human RNase H1 suggests that the enzyme fixes its position on the duplex via the 5′-RNA-3′-DNA terminus. Interestingly, the *in vitro* cleavage pattern observed for the enzyme is compatible with its proposed *in vivo* role, namely, the removal of RNA primers during DNA replication of the lagging strand. The average length of the RNA primer ranges from 7 to 14 nucleotides (42). Consequently, synthesis of the lagging strand results in chimeric sequences consisting of 7–14 ribonucleotides at the 5′ terminus with contiguous stretches of DNA extending in the 3′ direction. The positional preference observed for human RNase H1 (*i.e.* 8–12 residues from the 5′ terminus of the RNA) would suggest that cleavage of the chimeric lagging strand by RNase H1 would occur at or near the RNA-DNA junction. The removal of residual ribonucleotides following RNase H digestion has been shown to be performed by the endonuclease FEN1 (43).

Fig. 4 provides additional insight into the positional and sequence preferences of the enzyme. When there was a GU dinucleotide present in the correct position in the duplex, it was cleaved preferentially. When a GU dinucleotide was absent, AU was cleaved as well as other dinucleotides. For duplex G, both a GU and a GG dinucleotide were present within the preferred site, and in this case the GG dinucleotide was cleaved slightly more extensively than the GU dinucleotide. Clearly, additional duplexes of different sequences must be studied before definitive conclusions concerning the roles of various sequences within the preferred cleavage sites can be drawn.

In Fig. 5, the 3′ terminus of the RNA was labeled with [^{32}P]cytidine. In this case the same four nucleotides were cleaved as when the RNA was 5′-labeled (Fig. 5, *panels B* and *C*). However, the GU closer to the 3′ terminus of the RNA was cleaved at least as rapidly as the 5′-GU. Interestingly in studies on the partially purified enzyme, differences in the cleavage pattern were also observed when 5′-labeled substrates were compared with 3′-labeled substrates (6). At present, we have no explanation for this observation, but one possibility is that the presence of a 3′-phosphate on an oligonucleotide substrate affects the scanning mechanism the enzyme uses to select preferred positions for cleavage.

In a duplex comprised of RNA annealed to a chimeric oligonucleotide with an oligodeoxynucleotide center flanked by 2′-modified nucleotide wings, the cleavage by human RNase H1 was directed to the DNA-RNA portion of the duplex, as was observed for *E. coli* RNase H1 (18, 20). However, within this region, the preferred sites of cleavage for the human enzyme differed from *E. coli* RNase H1. *E. coli* RNase H1 preferentially cleaved at the ribonucleotide apposed to first 2′-modified nucleotide in the wing of antisense oligonucleotide at the 3′-end of the RNA (18). In contrast, the human enzyme preferentially cleaved at sites more centered within the gap until the gap was reduced to 5 nucleotides. Furthermore, the minimum gap size for the human enzyme was 5 nucleotides, whereas that of *E. coli* RNase H1 was 4 nucleotides (18). These differences in behavior suggest differences in the structures of the enzymes and their interactions with substrate that will require additional study.

We have reported that although *E. coli* RNase H1 degrades the heteroduplex substrate in a predominantly distributive manner, the enzyme displays modest 5′-3′ processivity. In contrast, human RNase H1 evidences no 5′-3′ or 3′-5′ processivity, suggesting that the human enzyme hydrolyzes the substrate in an exclusively distributive manner. The lack of processivity observed with the human RNase H1 may be a function of the significantly tighter binding affinity (Table IV), thereby reduc-

[2] L. B. Blyn, personal communication.

ing the ability of the enzyme to move on the substrate. Alternatively, human RNase H1 appears to fix its position on the substrate with respect to the 5′-RNA-3′-DNA terminus, and this strong positional preference may preclude cleavage of the substrate in a processive manner (Fig. 5). Thus, despite the facts that the enzymes are both metal-dependent endonucleases that result in cleavage products with 5′-phosphates (Fig. 5) and both can cleave single strand 3′-RNA overhangs (Fig. 5 and Ref. 20), these enzymes display substantial differences.

E. coli RNase H1 has been suggested to exhibit "binding directionality" with respect to the RNA of the substrate such that the primary binding region of the enzyme is positioned several nucleotides 5′ to the catalytic center (13). This results in cleavage sites being restricted from the 5′-RNA-3′-DNA end of a duplex and cleavage sites occurring at the 3′-RNA-5′-DNA end of the duplex and in 3′ single strand overhangs. The human enzyme behaves entirely analogously. Thus, we conclude that human RNase H1 likely has the same binding directionality as the *E. coli* enzyme.

Substrate Binding—RNA-RNA duplexes have been shown to adopt an A-form conformation (44, 45). Many 2′ modifications shift the sugar conformation into a 3′-endo pucker characteristic of RNA (9, 46–48). Consequently, when hybridized to RNA, the resulting duplex is A form, and this is manifested in a more stable duplex. 2′-fluoro oligonucleotides display duplex-forming properties most like RNA, whereas 2′-methoxy oligonucleotides result in duplex intermediate information between DNA-RNA and RNA-RNA duplexes (20).

The results shown in Table IV demonstrate that like the *E. coli* enzyme, human RNase H1 is a double strand RNA-binding protein. Moreover, it displays some ability to discriminate between various A-form duplexes (Table IV). The observation that the K_d for an RNA-2′-F duplex is equal to that for an RNA-RNA duplex suggests that 2′-hydroxy group is not required for binding to the enzyme. Nevertheless, we cannot exclude the possibility that bulkier 2′ modifications, e.g. 2′-methoxy or 2′-propyl, might sterically inhibit the binding of the enzyme as well as alter the A-form quality of the duplex. The human enzyme displays substantially greater affinity for all oligonucleotides than the *E. coli* enzyme, and this is reflected in a lower K_m for cleavable substrates (Tables III and IV). In addition, the tighter binding affinity observed for human RNase H1 may be responsible for the 20-fold lower V_{max} when compared with the *E. coli* enzyme. In this case, assuming that the *E. coli* and human enzymes exhibit similar catalytic rates (K_{cat}), then an increase in the binding affinity would result in a lower turnover rate and ultimately a lower V_{max}.

The principal substrate binding site in *E. coli* RNase H1 is thought to be a cluster of lysines that are believed to bind to the phosphates of the substrates (13). The interaction of the binding surface of the enzyme and substrate is believed to occur within the minor groove. This region is highly conserved in the human enzyme (16). In addition, eukaryotic enzymes contain an extra N-terminal region of variable length containing an abundance of basic amino acids (16, 17). This region is homologous with a double strand RNA binding motif and indeed in the *Saccharomyces cerevisiae* RNase H has been shown to bind to double strand RNA (17, 49). The N-terminal extension in human RNase H1 is longer than that in the *S. cerevisiae* enzyme and appears to correspond to a more complete double strand RNA binding motif. Consequently, the enhanced binding of human RNase H1 to various nucleic acids may be due to the presence of this additional binding site.

Biological Roles and Implications for Antisense Drug Design—As discussed previously, the positional preferences of human RNase H1 argue that the proposal that it may be involved in DNA replication may be correct (42). However, the lack of processivity would suggest that the enzyme is suboptimally designed for this task, but considering the involvement of FEN1 in DNA replication, processive cleavage of the RNA by RNase H may be unnecessary. Clearly, more work is required before any conclusions can be drawn.

Although RNase H enzymes have been suggested to be involved in the effects of DNA-like antisense drug, to date no studies have directly demonstrated this nor determined which isotypes may be involved. We now have the tools to begin to answer these questions. If human RNase H1 is involved, our studies suggest that excess single strand phosphorothioate oligonucleotides in cells would be highly inhibitory, resulting in loss of effectiveness at higher concentrations. Furthermore, the binding preference human RNase H1 displays for A-form duplexes suggests that binding of the enzyme would be enhanced by appropriate 2′ modifications. However, cleavage rates are lower in chimeric duplexes, so the design of optimal 2′-modified gap-mers may be challenging.

Clearly, if the positional and sequence preferences observed for oligonucleotide substrates were for RNA species bound to DNA-like antisense drugs, the implications would be substantial. For example, the placement of DNA gaps centered around a GU dinucleotide would be of value. Furthermore, since the positional preference of the enzyme was evident even when there were 5′- and 3′-RNA overhangs, positioning DNA gaps 8–12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex and creating a GU within that area could be beneficial. Also, locating antisense drugs at the 5′-end of an RNA should be of value. However, it is clear that many DNA-like antisense drugs bind to RNA species at sites distal from the 5′ terminus of the RNA and still result in loss of RNA, presumably via RNase H-mediated cleavage (50). Thus, much more work is required before conclusions can be drawn and the information can be used to design better antisense drugs.

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Antisense Research and Application

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CHAPTER 1

Basic Principles of Antisense Therapeutics

S.T. Crooke

A. Introduction

During the past few years, interest in developing antisense technology and in exploiting it for therapeutic purposes has been intense. Although progress has been gratifyingly rapid, the technology remains in its infancy and the questions that remain to be answered still outnumber the questions for which there are answers. Appropriately, considerable debate continues about the breadth of the utility of the approach and about the type of data required to "prove that a drug works through an antisense mechanism."

The objectives of this review are to provide a summary of recent progress, to assess the status of the technology, to place the technology in the pharmacological context in which it is best understood, and to deal with some of the controversies with regard to the technology and the interpretation of experiments.

B. Proof of Mechanism

I. Factors that May Influence Experimental Interpretations

Clearly, the ultimate biological effect of an oligonucleotide will be influenced by the local concentration of the oligonucleotide at the target RNA, the concentration of the RNA, the rates of synthesis and degradation of the RNA, the type of terminating mechanism, and the rates of the events that result in termination of the RNA's activity. At present, we understand essentially nothing about the interplay of these factors.

1. Oligonucleotide Purity

Currently, phosphorothioate oligonucleotides can be prepared consistently and with excellent purity (S.T. Crooke and Lebleu 1993). However, this has only been the case for the past 3–4 years. Prior to that time, synthetic methods were evolving and analytical methods were inadequate. In fact, our laboratory reported that different synthetic and purification procedures resulted in oligonucleotides that varied in cellular toxicity (R.M. Crooke 1991) and that potency varied from batch to batch. Though there are no longer synthetic problems with phosphorothioates, they, undoubtedly, complicated earlier

studies. More importantly, with each new analog class, new synthetic, purification, and analytical challenges are encountered.

2. Oligonucleotide Structure

Antisense oligonucleotides are designed to be single stranded. We now understand that certain sequences, e.g., stretches of guanosine residues, are prone to adopt more complex structures (Wyatt et al. 1994). The potential to form secondary and tertiary structures also varies as a function of the chemical class. For example, higher affinity 2'-modified oligonucleotides have a greater tendency to self hybridize, resulting in more stable oligonucleotide duplexes than would be expected based on rules derived from the behavior of oligodeoxynucleotides (S.M. Freier, unpublished results).

3. RNA Structure

RNA is structured. The structure of the RNA has a profound influence on the affinity of the oligonucleotide and on the rate of binding of the oligonucleotide to its RNA target (Freier 1993; Ecker 1993). Moreover, RNA structure produces asymmetrical binding sites that then result in very divergent affinity constants, depending on the position of oligonucleotide in that structure (Ecker 1993; Lima et al. 1992; Ecker et al. 1992). This in turn influences the optimal length of an oligonucleotide needed to achieve maximal affinity. We understand very little about how RNA structure and RNA protein interactions influence antisense drug action.

4. Variations in In Vitro Cellular Uptake and Distribution

Studies in several laboratories have clearly demonstrated that cells in tissue culture may take up phosphorothioate oligonucleotides via an active process and that the uptake of these oligonucleotides is highly variable depending on many conditions (R.M. Crooke 1991; S.T. Crooke et al. 1994). Cell type has a dramatic effect on total uptake, kinetics of uptake, and pattern of subcellular distribution. At present, there is no unifying hypothesis to explain these differences. Tissue culture conditions, such as the type of medium, degree of confluence, and the presence of serum, can all have enormous effects on uptake (S.T. Crooke et al. 1994). Oligonucleotide chemical class obviously influences the characteristics of uptake as well as the mechanism of uptake. Within the phosphorothioate class of oligonucleotides, uptake varies as a function of length, but not linearly. Uptake varies as a function of sequence, and stability in cells is also influenced by sequence (S.T. Crooke et al. 1994, 1995a).

Given the foregoing, it is obvious that conclusions about in vitro uptake must be very carefully made and generalizations are virtually impossible. Thus, before an oligonucleotide could be said to be inactive in vitro, it should be studied in several cell lines. Furthermore, while it may be absolutely correct that receptor-mediated endocytosis is a mechanism of uptake of phosphorothioate oligonucleotides (Loke et al. 1989), it is obviously simply unwarranted to generalize that all phosphorothioates are taken up by all cells in vitro primarily by receptor mediated endocytosis.

Finally, extrapolations from in vitro uptake studies to predict in vivo pharmacokinetic behavior are entirely inappropriate and misleading. There are now several lines of evidence in animals and man demonstrating that even after careful consideration of all in vitro uptake data, one cannot predict in vivo pharmacokinetics of the compounds, based on in vitro studies (S.T. Crooke et al. 1994; Cossum et al. 1993, 1994; Sands et al. 1995).

5. The Binding to and Effects of Binding to Nonnucleic Acid Targets

Phosphorothioate oligonucleotides tend to bind to many proteins and those interactions are influenced by many factors. Protein binding can influence cell uptake, distribution, metabolism and excretion. It may induce nonantisense effects that can be mistakenly interpreted as antisense or complicate the identification of an antisense mechanism. By inhibiting ribonuclease H (RNase H), protein binding may inhibit the antisense activity of some oligonucleotides. Finally, binding to proteins can certainly have toxicological consequences.

In addition to proteins, oligonucleotides may interact with other biological molecules, such as lipids or carbohydrates, and such interactions, like those with proteins, will be influenced by the chemical class of oligonucleotide studied. Unfortunately, essentially no data bearing on such interactions are currently available.

An especially complicated experimental situation is encountered in many in vitro antiviral assays. In these assays, high concentrations of drugs, viruses and cells are often coincubated. The sensitivity of each virus to nonantisense effects of oligonucleotides varies depending on the nature of the viral proteins and the characteristics of the oligonucleotides (Cowsert 1993; Cowsert et al. 1993). This has resulted in considerable confusion. In particular, for HIV, herpes simplex viruses, cytomegaloviruses, and influenza virus, nonantisense effects have been so dominant that identifying oligonucleotides that work via an antisense mechanism has been difficult. Given the artificial character of such assays, it is difficult to know whether nonantisense mechanisms would be as dominant in vivo or result in antiviral activity.

6. Terminating Mechanisms

It has been amply demonstrated that oligonucleotides may employ several terminating mechanisms. The dominant terminating mechanism is influenced by RNA receptor site, oligonucleotide chemical class, cell type, and probably many other factors (S.T. Crooke 1995b). Obviously, as variations in terminating mechanism may result in significant changes in antisense potency and studies have shown significant variations from cell type to cell type in vitro, it

is essential that the terminating mechanism be well understood. Unfortunately, at present, our understanding of terminating mechanisms remains rudimentary.

7. Effects of "Control Oligonucleotides"

A number of types of control oligonucleotides have been used, including randomized oligonucleotides. Unfortunately, we know little to nothing about the potential biological effects of such "controls" and the more complicated a biological system and test the more likely that "control" oligonucleotides may have activities that complicate interpretations. Thus, when a control oligonucleotide displays a surprising activity, the mechanism of that activity should be explored carefully before concluding that the effects of the "control oligonucleotide" prove that the activity of the putative antisense oligonucleotide are not due to an antisense mechanism.

8. Kinetics of Effects

Many rate constants may affect the activities of antisense oligonucleotides, e.g., the rate of synthesis and degradation of the target RNA and its protein, the rates of uptake into cells, the rates of distribution, extrusion, and metabolism of an oligonucleotide in cells, and similar pharmacokinetic considerations in animals. Despite this, relatively few time courses have been reported, and in vitro studies have been reported that range from a few hours to several days. In animals, we have a growing body of information on pharmacokinetics, but in most studies reported to date, the doses and schedules were chosen arbitrarily and, again, little information on duration of effect and onset of action has been presented. Clearly, more careful kinetic studies are required and rational in vitro and in vivo dose schedules must be developed.

II. Recommendations

1. Positive Demonstration of Antisense Mechanism and Specificity

Until more is understood about how antisense drugs work, it is essential to positively demonstrate effects consistent with an antisense mechanism. For RNase H activating oligonucleotides, northern blot analysis showing selective loss of the target RNA is the best choice and many laboratories are publishing reports in vitro and in vivo of such activities (Chiang et al. 1991; Dean and McKay 1994; Skorski et al. 1994; Hijiya et al. 1994). Ideally, a demonstration that closely related isotypes are unaffected should be included. In brief, then, for proof of mechanism, the following steps are recommended:

1. Perform careful dose response curves in vitro using several cell lines and methods of in vitro delivery
2. Correlate the rank order potency in vivo with that observed in vitro after thorough dose response curves are generated in vivo

3. Perform careful "gene walks" for all RNA species and oligonucleotide chemical classes.
4. Perform careful time courses before drawing conclusions about potency
5. Directly demonstrate proposed mechanism of action by measuring the target RNA and/or protein
6. Evaluate specificity and therapeutic indices via studies on closely related isotypes and with appropriate toxicological studies
7. Perform sufficient pharmacokinetics to define rational dosing schedules for pharmacological studies
8. When control oligonucleotides display surprising activities, determine the mechanisms involved

C. Molecular Mechanisms of Antisense Drugs

1. Occupancy Only Mediated Mechanisms

Classic competitive antagonists are thought to alter biological activities because they bind to receptors, preventing natural agonists from binding and in this way inducing normal biological processes. Binding of oligonucleotides to specific sequences may inhibit the interaction of the RNA with proteins, other nucleic acids, or other factors required for essential steps in the intermediary metabolism of the RNA or its utilization by the cell.

1. Inhibition of Splicing

A key step in the intermediary metabolism of most mRNA molecules is the excision of introns. These "splicing" reactions are sequence specific and require the concerted action of spliceosomes. Consequently, oligonucleotides that bind to sequences required for splicing may prevent binding of these factors or physically prevent the required cleavage reactions. This should result in inhibition of the production of the mature mRNA. Although there are several examples of oligonucleotides directed to splice junctions, none of the studies present data showing inhibition of RNA processing, accumulation of splicing intermediates, or a reduction in mature mRNA. Nor are there published data in which the structure of the RNA at the splice junction was probed and the oligonucleotides demonstrated to hybridize to the sequences for which they were designed (McManaway et al. 1990; Kulick et al. 1990; Zamecnik et al. 1986; Smith et al. 1986). Activities have been reported for anti-*c-myc* and antiviral oligonucleotides with phosphodiester, methylphosphonate, and phosphorothioate backbones. Very recently, an oligonucleotide was reported to induce alternative splicing in a cell-free splicing system and, in this system, RNA analyses confirmed the putative mechanism (Dominski and Kole 1993).

In our laboratory, we have attempted to characterize the factors that determine whether splicing inhibition is effected by an antisense mechanism

List of Abbreviations

CMV	cytomegalovirus
Ha-ras	Harvey *ras*
HIV	human immune deficiency virus
HPV	human papillomavirus
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
IL	interleukin
mRNA	messenger RNA
NK	natural killer
PKC	protein kinase C
PNA	peptide nucleic acid
PTHrP	parathyroid hormone-related peptide
RT	reverse transcriptase
TAR	transactivator response element
T_m	melting transition

(Hodges and Crooke 1995). To this end, a number of luciferase-reporter plasmids containing various introns were constructed and transfected into HeLa cells. Then the effects of antisense drugs designed to bind to various sites were characterized. The effects of RNase H-competent oligonucleotides were compared to those of oligonucleotides that do not serve as RNase H substrates. The major conclusions from this study were as follows. First, most of the earlier studies in which splicing inhibition was reported were probably due to nonspecific effects. Second, less effectively spliced introns are better targets than those with strong consensus splicing signals. Third, the 3'-splice site and branchpoint are usually the best sites to which to target to the oligonucleotide to inhibit splicing. Fourth, RNase H-competent oligonucleotides are usually more potent than even higher affinity oligonucleotides that inhibit by occupancy only.

2. Translational Arrest

Many oligonucleotides have been designed to arrest translation of targeted protein by binding to the translation initiation codon. The positioning of the initiation codon within the area of complementarity of the oligonucleotide and the length of oligonucleotide used have varied considerably. Again, unfortunately, only in relatively few studies have the oligonucleotides, in fact, been shown to bind to the sites for which they were designed, and data that directly support translation arrest as the mechanism have been lacking.

Target RNA species that have been reported to be inhibited by a translational arrest mechanism include HIV, vesicular stomatitis virus (VSV), N-*myc* and a number of normal cellular genes (Agrawal et al. 1988; LeMaitre et al. 1987; Rosolen et al. 1990; Vasanthakumar and Ahmed 1989; Saurlati et al. 1991; Zheng et al. 1989; Maier et al. 1990). In our laboratories, we have shown that a significant number of targets may be inhibited by binding to translation initiation codons. For example, ISIS 1082 hybridizes to the AUG codon (gene) the UL13 gene of herpes virus types 1 and 2. RNase H studies confirmed that it binds selectively in this area. In vitro protein synthesis studies confirmed that it inhibited the synthesis of the UL13 protein and studies in HeLa cells showed that it inhibited the growth of herpes type 1 and type 2 with IC_{50} of 200–400 nM by translation arrest (Mirabelli et al. 1991). Similarly, ISIS 1753, a 20-mer phosphorothioate complementary to the translation initiation codon and surrounding sequences of the *E2* gene of bovine papilloma virus, was highly effective and its activity was shown to be due to translation arrest. ISIS 2105, a 20-mer phosphorothioate complementary to the same region in human papilloma virus, was shown to be a very potent inhibitor. Compounds complementary to the translation initiation codon of the E2 gene were the most potent of the more than 50 compounds studied complementary to various other regions in the RNA (Cowsert et al. 1993). We have also shown inhibition of translation of a number of other mRNA species by compounds designed to bind to the translation codon.

In conclusion, translation arrest represents an important mechanism of action for antisense drugs. A number of examples purporting to employ this mechanism have been reported, and recent studies on several compounds have provided data that unambiguously demonstrate that this mechanism can result in potent antisense drugs. However, very little is understood about the precise events that lead to translation arrest.

3. Disruption of Necessary RNA Structure

RNA adopts a variety of three-dimensional structures induced by intramolecular hybridization, the most common of which is the stem loop. These structures play crucial roles in a variety of functions. They are used to provide additional stability for RNA and as recognition motifs for a number of proteins, nucleic acids, and ribonucleoproteins that participate in the intermediary metabolism and activities of RNA species. Thus, given the potential general activity of the mechanism, it is surprising that occupancy-based disruption RNA has not been more extensively exploited.

As an example, we designed a series of oligonucleotides that bind to the important stem loop present in all RNA species in HIV, the TAR element. We synthesized a number of oligonucleotides designed to disrupt TAR and showed that several did indeed bind to TAR, disrupt the structure, and inhibit TAR-mediated production of a reporter gene (Vickers et al. 1991). Furthermore, general rules useful in disrupting stem-loop structures were also developed (Ecker et al. 1992).

Although designed to induce relatively nonspecific cytotoxic effects, two other examples are noteworthy. Oligonucleotides designed to bind to a 17-

nucleotide loop in Xenopus 28 S RNA required for ribosome stability and protein synthesis inhibited protein synthesis when injected into Xenopus oocytes (Saxena and Ackerman 1990). Similarly, oligonucleotides designed to bind to highly conserved sequences in 5.8S RNA inhibited protein synthesis in rabbit reticulocyte and wheat germ systems (Walker et al. 1990).

II. Occupancy Activated Destabilization

RNA molecules regulate their own metabolism. A number of structural features of RNA are known to influence stability, various processing events, subcellular distribution and transport. It is likely that, as RNA intermediary metabolism is better understood, many other regulatory features and mechanisms will be identified.

1. 5' Capping

A key early step in RNA processing is 5' capping (Fig. 1). This stabilizes pre-mRNA and is important for the stability of mature mRNA. It is also important in binding to the nuclear matrix and transport of mRNA out of the nucleus. As the structure of the cap is unique and understood, it presents an interesting target.

Several oligonucleotides that bind near the cap site have been shown to be active, presumably by inhibiting the binding of proteins required to cap the

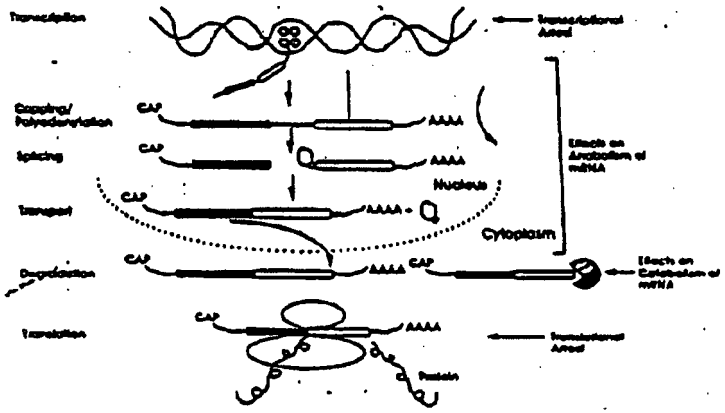

Fig. 1. RNA processing

RNA. For example, the synthesis of SV40 T-antigen was reported to be most sensitive to an oligonucleotide linked to polylysine and targeted to the 5' cap site of RNA (Wickstrom et al. 1989). However, once again, in no published study has this putative mechanism been rigorously demonstrated. In fact, in no published study have the oligonucleotides been shown to bind to the sequences for which they were designed.

In our laboratory, we have designed oligonucleotides to bind to 5' cap structures and reagents to specifically cleave the unique 5' cap linkage (Baker 1993). These studies demonstrate that 5'-cap targeted oligonucleotides were capable of inhibiting the binding of the eukaryotic translation initiation factor 4a (eIF-4a) (Baker et al. 1992).

2. Inhibition of 3' Polyadenylation

In the 3'-untranslated region of pre-mRNA molecules, there are sequences that result in the post-transcriptional addition of long (hundreds of nucleotides) tracts of polyadenylate. Polyadenylation stabilizes mRNA and may play other roles in the intermediary metabolism of RNA species. Theoretically, interactions in the 3'-terminal region of pre-mRNA could inhibit polyadenylation and destabilize the RNA species. Although there are a number of oligonucleotides that interact in the 3'-untranslated region and display antisense activities, to date, no study has reported evidence for alterations in polyadenylation (Chiang et al. 1991).

III. Other Mechanisms

In addition to 5' capping and 3' adenylation, there are clearly other sequences in the 5'- and 3'-untranslated regions of mRNA that affect the stability of the molecules. Again, there are a number of antisense drugs that may work by these mechanisms.

Zamecnik and Stephenson (1978) reported that a 13 mer targeted to untranslated 3'- and 5'-terminal sequences in Rous sarcoma virus was active. Oligonucleotides conjugated to an acridine derivative and targeted to the terminal sequence in type A influenza viruses were reported to be active. Against several RNA targets, studies in our laboratories have shown that sequences in the 3'-untranslated region of RNA molecules are often hypersensitive (Zerial et al. 1987; Thiong et al. 1989; Helene and Toulme 1990). For example, ISIS 1939 is a 20-mer phosphorothioate that binds to and appears to disrupt a predicted stem-loop structure in the 3'-untranslated region of the mRNA for ICAM and is a potent antisense inhibitor. However, inasmuch as a 2'-methoxy analog of ISIS 1939 was much less active, it is likely that in addition to destabilization to cellular nucleolytic activity, activation of RNase H (see below) is also involved in the activity of ISIS 1939 (Chiang et al. 1991).

IV. Activation of RNase H

RNase H is an ubiquitous enzyme that degrades the RNA strand of an RNA–DNA duplex. It has been identified in organisms as diverse as viruses and human cells (Crouch and Dirksen 1985). At least two classes of RNase H have been identified in eukaryotic cells. Multiple enzymes with RNase H activity have been observed in prokaryotes (Crouch and Dirksen 1985).

Although RNase H is involved in DNA replication, it may play other roles in the cell and is found in the cytoplasm as well as the nucleus (Crum et al. 1988). However, the concentration of the enzyme in the nucleus is thought to be greater and some of the enzyme found in cytoplasmic preparations may be due to nuclear leakage.

RNase H activity is quite variable in cells. It is absent or minimal in rabbit reticulocytes but present in wheat germ extracts (Crouch and Dirksen 1985; Hazuttle et al. 1986). In HL-60 cells, for example, the level of activity in undifferentiated cells is greatest; it is relatively high in DMSO and Vitamin D differentiated cells and much lower in phorbol myristic acid (PMA) -differentiated cells (G. Hoke, unpublished data).

The precise recognition elements for RNase H are not known. However, it has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (Donis-Keller 1979). Changes in the sugar influence RNase H activation as sugar modifications that result in RNA-like oligonucleotides, e.g., 2′ fluoro or 2′ methoxy do not appear to serve as substrates for RNase H (Kawasaki et al. 1993; Sproat et al. 1989). Alterations in the orientation of the sugar to the base can also affect RNase H activation as α-oligonucleotides are unable to induce RNase H or may require parallel annealing (Morvan et al. 1991; Gagnor et al. 1989). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates do not activate RNase H (Maher et al. 1989; Miller 1989). In contrast, phosphorothioates are excellent substrates (Mirabelli et al. 1991; Stein and Cheng 1993; Cazenave et al. 1989). In addition, chimeric molecules have been studied as oligonucleotides that bind to RNA and activate RNase H (Quartin et al. 1989; Furdon et al. 1989). For example, oligonucleotides comprised of wings of 2′-methoxy phosphorothioates and a five-base gap of deoxyoligonucleotides bind to their target RNA and activate RNase H (Quartin et al. 1989; Furdon et al. 1989). Furthermore, a single ribonucleotide in a sequence of deoxyribonucleotides was shown to be sufficient to serve as a substrate for RNase H when bound to its complementary oligodeoxynucleotide (Eder and Walder 1991).

That it is possible to take advantage of chimeric oligonucleotides designed to activate RNase H that have greater affinity for their RNA receptors and to enhance specificity has also been demonstrated (Monia et al. 1993; Giles and Tidd 1992). In a recent study, RNase H mediated cleavage of target transcript was much more selective when oligodeoxynucleotides comprised of methylphosphonate oligodeoxynucleotide wings and phosphodiester gaps were compared to full phosphodiester oligonucleotides (Giles and Tidd 1992).

Despite the information about RNase H and the demonstration that many oligonucleotides may activate RNase H in lysate and purified enzyme assays, relatively little is yet known about the role of structural features in RNA targets in activating RNase H (Walder and Walder 1988; Minshull and Hunt 1986; Gagnor et al. 1987). In fact, direct proof that RNase H activity is, in fact, the mechanism of action of oligonucleotides in cells is to a large extent lacking.

Recent studies in our laboratories provide additional, albeit indirect, insights into these questions. ISIS 1939 is a 20-mer phosphorothioate complementary to a sequence in the 3′-untranslated region of ICAM-1 RNA (Chiang et al. 1991). It inhibits ICAM production in human umbilical vein endothelial cells, and northern blots demonstrate that ICAM-1 mRNA is rapidly degraded. A 2′-methoxy analog of ISIS 1939 displays higher affinity for the RNA than the phosphorothioate and is stable in cells, but inhibits ICAM-1 protein production much less potently than ISIS 1939. It is likely that ISIS 1939 destabilizes the RNA and activates RNase H. In contrast, ISIS 1570, an 18-mer phosphorothioate that is complementary to the translation initiation codon of the ICAM-1 message inhibited production of the protein, but caused no degradation of the RNA. Thus, two oligonucleotides that are capable of activating RNase H had different effects, depending on the site in the mRNA at which they bound (Chiang et al. 1991).

A more direct demonstration that RNase H is likely a key factor in the activity of many antisense oligonucleotides was provided by studies in which reverse-ligation polymerase chain reaction (PCR) was used to identify cleavage products from *bcr-abl* mRNA in cells treated with phosphorothioate oligonucleotides (Giles et al. 1995).

Given the emerging role of chimeric oligonucleotides with modifications in the 3′ and 5′ wings designed to enhance affinity for the target RNA and nuclease stability and a DNA-type gap to serve as a substrate for RNase H, studies focused on understanding the effects of various modifications on the efficiency of the enzyme(s) are also of considerable importance. In one such study on *Escherichia coli* RNase H, we have recently reported that the enzyme displays minimal sequence specificity and is processive. When a chimeric oligonucleotide with 2′-modified sugars in the wings was hybridized to the RNA, the initial site of cleavage was the nucleotide adjacent to the methoxy-deoxy junction closest to the 3′ end of the RNA substrate. The initial rate of cleavage increased as the size of the DNA gap increased, and the efficiency of the enzyme was considerably less against an RNA target duplexed with a chimeric antisense oligonucleotide than a full DNA-type oligonucleotide (S.T. Crooke et al. 1995).

In subsequent studies, we have evaluated in more detail the interactions of antisense oligonucleotides with structured and unstructured targets and the impacts of these interactions on RNase H (Lima and Crooke 1997). Using a

series of noncleavable substrates and Michaelis-Menten analyses, we were able to evaluate both binding and cleavage. We showed that, in fact, *E. coli* RNase H1 is a double-strand RNA binding protein. The K_d for an RNA duplex was 1.6 μM; the K_d for a DNA duplex was 176 μM; and the K_d for single-strand DNA was 942 μM. In contrast, the enzyme could only cleave RNA in an RNA–DNA duplex. Any 2′ modification in the antisense drug at the cleavage site inhibited cleavage, but significant charge reduction and 2′ modifications were tolerated at the binding site. Finally, placing a positive charge (e.g., 2′ propoxyamine) in the antisense drug reduced affinity and cleavage.

We have also examined the effects of antisense oligonucleotide-induced RNA structures on the activity of *E. Coli* RNase H1 (Lima et al., in press). Any structure in the duplex substrate was found to have a significant negative effect on the cleavage rate. Further, cleavage of selected sites was inhibited entirely, and this was explained by the steric hindrance imposed by the RNA loop traversing either the minor or major grooves or the heteroduplex.

V. Activation of Double-Strand RNase

By using phosphorothioate oligonucleotides with 2′ modified wings and a ribonucleotide center, we have shown that mammalian cells contain enzymes that can cleave double-strand RNAs (Wu et al., submitted). This is an important step forward because it adds to the repertoire of intracellular enzymes that may be used to cleave target RNAs and because chimeric oligonucleotides 2′ modified wings and oligoribonucleotide gaps have higher affinity for RNA targets than chimerns with oligodeoxynucleotide gaps.

D. Characteristics of Phosphorothioate Oligodeoxynucleotides

I. Introduction

Of the first generation oligonucleotide analogs, the class that has resulted in the broadest range of activities and about which the most is known is the phosphorothioate class. Phosphorothioate oligonucleotides were first synthesized in 1969 when a poly rI-rC phosphorothioate was synthesized (De Clercq et al. 1969). This modification clearly achieves the objective of increased nuclease stability. In this class of oligonucleotides, one of the oxygen atoms in the phosphate group is replaced with a sulfur. The resulting compound is negatively charged, is chiral at each phosphorothioate, and much more resistant to nucleases than the parent phosphodiester (Cohen 1993).

II. Hybridization

The hybridization of phosphorothioate oligonucleotides to DNA and RNA has been thoroughly characterized (Crooke and Lebleu 1993; Crooke 1992; R.M. Crooke 1993a). The melting transition (T_m) of a phosphorothioate oligodeoxynucleotide for RNA is approximately 0.5°C less per nucleotide than for a corresponding phosphodiester oligodeoxynucleotide. This reduction in T_m per nucleotide is virtually independent of the number of phosphorothioate units substituted for phosphodiesters. However, sequence context has some influence as the ΔT_m can vary from −0.3°C to 1.0°C depending on sequence. Compared to RNA and RNA duplex formation, a phosphorothioate oligodeoxynucleotide has a T_m approximately 2.2°C lower per unit (Freier 1993). This means that to be effective in vitro, phosphorothioate oligodeoxynucleotides must typically be 17–20 mer in length and that invasion of double-stranded regions in RNA is difficult (Lima et al. 1992; Vickers et al. 1991; Monia et al. 1992, 1993).

Association rates of phosphorothioate oligodeoxynucleotide to unstructured RNA targets are typically 10^6–10^7 $M^{-1}S^{-1}$, independent of oligonucleotide length or sequence (Freier 1993; Lima et al. 1992). Association rates to unstructured RNA targets can vary from 10^3 to 10^6 $M^{-1}S^{-1}$, depending on the structure of the RNA, the site of binding in the structure, and other factors (Freier 1993). Put in another way, association rates for oligonucleotides that display acceptable affinity constants are sufficient to support biological activity at therapeutically achievable concentrations.

The specificity of hybridization of phosphorothioate oligonucleotides is, in general, slightly greater than that of phosphodiester analogs. For example, a T-C mismatch results in a 7.7°C or 12.8°C reduction in T_m, respectively, for a phosphodiester or phosphorothioate oligodeoxynucleotide 18 nucleotides in length with the mismatch centered (Freier 1993). Thus, from this perspective, the phosphorothioate modification is quite attractive.

III. Interactions with Proteins

Phosphorothioate oligonucleotides bind to proteins. The interactions with proteins can be divided into nonspecific, sequence specific, and structure-specific binding events, each of which may have different characteristics and effects. Nonspecific binding to a wide variety of proteins has been demonstrated. Exemplary of this type of binding is the interaction of phosphorothioate oligonucleotides with serum albumin. The affinity of such interactions is low. The K_d for albumin is approximately 200 μM, and thus in a similar range with aspirin or penicillin (S.T. Crooke et al. 1996; Foss and Hall 1969). Furthermore, in this study, no competition between phosphorothioate oligonucleotides and several drugs that bind to bovine serum albumin was observed. In this study, binding and competition were determined in an assay in which electrospray mass spectrometry was used. In

contrast, in a study in which an equilibrium dissociation constant was derived from an assay using albumin loaded on a CH-sephadex column, the K_d ranged from 1 to 5×10^{-6} M for bovine serum albumin and from 2 to 3×10^{-6} M for human serum albumin. Moreover, warfarin and indomethacin were reported to compete for binding to serum albumin (Srinivasan et al. 1995). Clearly, much more work is required before definitive conclusions can be drawn.

Phosphorothioate oligonucleotides can interact with nucleic acid binding proteins such as transcription factors and single-strand nucleic acid binding proteins. However, very little is known about these binding events. Additionally, it has been reported that phosphorothioates bind to an 80-KDa membrane protein that was suggested to be involved in cellular uptake processes. (Loke et al. 1989). However, again, little is known about the affinities, sequence or structure specificities of these putative interactions. More recently interactions with 30-KDa and 46-KDa surface proteins in T15 mouse fibroblasts were reported (Hawley and Gibson 1996).

Phosphorothioates interact with nucleases and DNA polymerases. These compounds are slowly metabolized by both endo- and exonucleases and inhibit these enzymes (R.M. Crooke et al. 1995; S.T. Crooke 1992). The inhibition of these enzymes appears to be competitive, and this may account for some early data suggesting that phosphorothioates were almost infinitely stable to nucleases. In these studies, the oligonucleotide to enzyme ratio was very high and, thus, the enzyme was inhibited. Phosphorothioates also bind to RNase H when in an RNA–DNA duplex, and the duplex serves as a substrate for RNase H (Gao et al. 1992). At higher concentrations, presumably by binding as a single strand to RNase H, phosphorothioates inhibit the enzyme (S.T. Crooke et al. 1995; S.T. Crooke 1992). Again, the oligonucleotides appear to be competitive antagonists for the DNA–RNA substrate.

Phosphorothioates have been shown to be competitive inhibitors of DNA polymerase α and β with respect to the DNA template, and noncompetitive inhibitors of DNA polymerases γ and δ (Gao et al. 1992). Despite this inhibition, several studies have suggested that phosphorothioates might serve as primers for polymerases and be extended (Stein and Cheng 1993; S.T. Crooke 1995a; Agrawal et al. 1991). In our laboratories, we have shown extensions of 2–3 nucleotides only. At present, a full explanation as to why no longer extensions are observed is not available.

Phosphorothioate oligonucleotides have been reported to be competitive inhibitors for HIV-reverse transcriptase and to inhibit reverse transcriptase (RT)-associated RNase H activity (Majumdar et al. 1989; Cheng et al. 1991). They have been reported to bind to the cell surface protein, CD4, and to protein kinase C (Stein et al. 1991). Various viral polymerases have also been shown to be inhibited by phosphorothioates (Stein and Cheng 1993). Additionally, we have shown potent, nonsequence specific inhibition of RNA splicing by phosphorothioates (Hooges and Crooke 1995).

Like other oligonucleotides, phosphorothioates can adopt a variety of secondary structures. As a general rule, self-complementary oligonucleotides

are avoided, if possible, to avoid duplex formation between oligonucleotides. However, other structures that are less well understood can also form. For example, oligonucleotides containing runs of guanosines can form tetrameric structures called G (guanosine) quartets, and these appear to interact with a number of proteins with relatively greater affinity than unstructured oligonucleotides (Wyatt et al. 1994).

In conclusion, phosphorothioate oligonucleotides may interact with a wide range of proteins via several types of mechanisms. These interactions may influence the pharmacokinetic, pharmacologic, and toxicologic properties of these molecules. They may also complicate studies on the mechanism of action of these drugs, and may, in fact, obscure an antisense activity. For example, phosphorothioate oligonucleotides were reported to enhance lipopolysaccharide-stimulated synthesis of tumor necrosis factor (Hartmann et al. 1996). This would obviously obscure antisense effects on this target.

IV. Pharmacokinetic Properties

To study the pharmacokinetics of phosphorothioate oligonucleotides, a variety of labeling techniques have been used. In some cases, 3′- or 5′ ^{32}P-end-labeled or fluorescently labeled oligonucleotides have been used in in vitro or in vivo studies. These are probably less satisfactory than internally labeled compounds because terminal phosphates are rapidly removed by phosphatases and fluorescently labeled oligonucleotides have physicochemical properties that differ from the unmodified oligonucleotides. Consequently, either uniformly (Cowsert et al. 1993) S-labeled, or base-labeled phosphorothioates are preferable for pharmacokinetic studies. In our laboratories, a tritium exchange method that labels a slowly exchanging proton at the C8 position in purines was developed and proved to be quite useful (Graham et al. 1993). Very recently, a method that added radioactive methyl groups via *S*-adenosylmethionine has also been successfully used (Sands et al. 1994). Finally, advances in extraction, separation and detection methods have resulted in methods that provide excellent pharmacokinetic analyses without radiolabeling (S.T. Crooke et al. 1996).

1. Nuclease Stability

The principle metabolic pathway for oligonucleotides is cleavage via endo- and exonucleases. Phosphorothioate oligonucleotides, while quite stable to various nucleases are competitive inhibitors of nucleases (S.T. Crooke 1995b; Gao et al. 1992; Hoke et al. 1991; Wickstrom 1986; Campbell et al. 1990). Consequently, the stability of phosphorothioate oligonucleotides to nucleases is probably a bit less than initially thought, as high concentrations (that inhibited nucleases) of oligonucleotides were employed in the early studies. Similarly, phosphorothioate oligonucleotides are degraded slowly by cells in tissue culture with a half-life of 12–24 h and are slowly metabolized in animals (S.T.

Crooke 1995b; Cossum et al. 1993; Hoke et al. 1991). The pattern of metabolites suggests primarily exonuclease activity with perhaps modest contributions by endonucleases. However, a number of lines of evidence suggest that, in many cells and tissues, endonucleases play an important role in the metabolism of oligonucleotides. For example, 3'- and 5'-modified oligonucleotides with phosphodiester backbones have been shown to be relatively rapidly degraded in cells and after administration to animals (Sands et al. 1995; Miyao et al. 1995). Thus, strategies in which oligonucleotides are modified at only the 3' and 5' terminus as a means of enhancing stability have not proven to be successful.

2. In Vitro Cellular Uptake

Phosphorothioate oligonucleotides are taken up by a wide range of cells in vitro (R.M. Crooke 1991, 1993a; R.M. Crooke et al. 1995; Gao et al. 1992; Neckers 1993). In fact, uptake of phosphorothioate oligonucleotides into a prokaryote, *Vibrio parahaemolyticus*, has been reported, as has uptake into *Schistosoma mansoni* (Chrisey et al. 1993; Tao et al. 1995). Uptake is time and temperature dependent. It is also influenced by cell type, cell-culture conditions, media and sequence, and length of the oligonucleotide (R.M. Crooke et al. 1995). No obvious correlation between the lineage of cells, whether the cells are transformed or whether they are virally infected and uptake has been identified (S.T. Crooke 1995a). Nor are the factors that result in differences in uptake of different sequences of oligonucleotide understood. Although several studies have suggested that receptor-mediated endocytosis may be a significant mechanism of cellular uptake, the data are not yet compelling enough to conclude that receptor-mediated endocytosis accounts for a significant portion of the uptake in most cells (Lake et al. 1989).

Numerous studies have shown that phosphorothioate oligonucleotides distribute broadly in most cells once taken up (S.T. Crooke 1995a; R.M. Crooke 1993a). Again, however, significant differences in subcellular distribution between various types of cells have been noted.

Cationic lipids and other approaches have been used to enhance uptake of phosphorothioate oligonucleotides in cells that take up little oligonucleotide in vitro (Bennett et al. 1992, 1993; Quattrone et al. 1994). Again, however, there are substantial variations from cell type to cell type. Other approaches to enhanced intracellular uptake in vitro have included streptolysin O treatment of cells and the use of dextran sulfate and other liposome formulations as well as physical means such as microinjections (S.T. Crooke 1995a; Giles et al. 1995; Wang et al. 1995).

3. In Vivo Pharmacokinetics

Phosphorothioate oligonucleotides bind to serum albumin and α_2-macroglobulin. The apparent affinity for albumin is quite low (200–400 μM) and comparable to the low-affinity binding observed for a number of drugs, e.g., aspirin and penicillin (S.T. Crooke et al. 1996; Joos and Hall 1969; Srinivasan et al. 1995). Serum protein binding, therefore, provides a repository for these drugs and prevents rapid renal excretion. As serum-protein binding is saturable at higher doses, intact oligomer may be found in urines (Agrawal et al. 1991; Iversen 1991). Studies in our laboratory suggest that in rats, oligonucleotides administered intravenously at doses of 15–20 mg/kg saturate the serum protein binding capacity (J. Leeds, unpublished data).

Phosphorothioate oligonucleotides are rapidly and extensively absorbed after parenteral administration. For example, in rats, after an intradermal dose of 3.6 mg/kg of ^{14}C-labeled ISIS 2105, a 20-mer phosphorothioate, approximately 70% of the dose was absorbed within 4 h and total systemic bioavailability was in excess of 90% (Cossum et al. 1994). After intradermal injection in man, absorption of ISIS 2105 was similar to that observed in rats (S.T. Crooke et al. 1994). Subcutaneous administration to rats and monkeys results in somewhat lower bioavailability and greater distribution to lymph, as would be expected (J. Leeds, unpublished observations).

Distribution of phosphorothioate oligonucleotides from blood after absorption or intravenous administration is extremely rapid. We have reported distribution half-lives of less than 1 h, and similar data have been reported by others (Cossum et al. 1993, 1994; Agrawal et al. 1991; Iversen 1991). Blood and plasma clearance is multiexponential with a terminal elimination half-life from 40 to 60 h in all species except man. In man, the terminal elimination half-life may be somewhat longer (S.T. Crooke et al. 1994).

Phosphorothioates distribute broadly to all peripheral tissues. Liver, kidney, bone marrow, skeletal muscle, and skin accumulate the highest percentage of a dose, but other tissues display small quantities of drug (Cossum et al. 1993, 1994). No evidence of significant penetration of the blood–brain barrier has been reported. The rates of incorporation and clearance from tissues vary as a function of the organ studied, with liver accumulating drug most rapidly (20% of a dose within 1–2 h) and other tissues accumulating drug more slowly. Similarly, elimination of drug is more rapid from liver than any other tissue, e.g., terminal half-life from liver: 62 h, from renal medulla: 156 h. The distribution into the kidney has been studied more extensively and drug shown to be present in Bowman's capsule, the proximal convoluted tubule, the brush border membrane, and within renal tubular epithelial cells (Rappaport et al. 1995). The data suggest that the oligonucleotides are filtered by the glomerulus, then reabsorbed by the proximal convoluted tubule epithelial cells. Moreover, the authors suggest that reabsorption might be mediated by interactions with specific proteins in the brush border membranes.

At relatively low doses, clearance of phosphorothioate oligonucleotides is due primarily to metabolism (Cossum et al. 1993, 1994; Iversen 1991). Metabolism is mediated by exo- and endonucleases that result in shorter oligonucleotides and, ultimately, nucleosides that are degraded by normal metabolic pathways. Although no direct evidence of base excision modification has been reported, these are theoretical possibilities that may occur. In

one study, a larger molecular weight radioactive material was observed in urine, but not fully characterized (Agrawal et al. 1991). Clearly, the potential for conjugation reactions and extension of oligonucleotides via these drugs serving as primers for polymerases must be explored in more detail. In a very thorough study, 20 nucleotide phosphodiester and phosphorothioate oligonucleotides were administered intravenously at a dose of 6 mg/kg to mice. The oligonucleotides were internally labeled with ^3H-CH_3 by methylation of an internal deoxycytidine residue using HhaI methylase and *S*-(3H) adenosylmethionine (Sands et al. 1994). The observations for the phosphorothioate oligonucleotide were entirely consistent with those made in our studies. Additionally, in this paper, autoradiographic analyses showed drug in renal cortical cells (Sands et al. 1994).

One study of prolonged infusions of a phosphorothioate oligonucleotide to human beings has been reported (Bayever et al. 1993). In this study, five patients with leukemia were given 10-day intravenous infusions at a dose of 0.05 mg/kg per hour. Elimination half-lives reportedly varied from 5.9 to 14.7 days. Urinary recovery of radioactivity was reported to be 30%–60% of the total dose, with 30% of the radioactivity being intact drug. Metabolites in urine included both higher and lower molecular weight compounds. In contrast, when GEM-91 (a 25-mer phosphorothioate oligodeoxynucleotide) was administered to humans as a 2-h, I.V. infusion at a dose of 0.1 mg/kg, a peak plasma concentration of 295.8 mg/ml was observed at the cessation of the infusion. Plasma clearance of total radioactivity was biexponential, with initial and terminal elimination half-lives of 0.18 and 26.71 h, respectively. However, degradation was extensive and intact drug pharmacokinetic models were not presented. Nearly 50% of the administered radioactivity was recovered in urine, but most of the radioactivity represented degradates. In fact, no intact drug was found in the urine at any time (Zhang et al. 1995a).

In a more recent study in which the level of intact drug was carefully evaluated using capillary gel electrophoresis, the pharmacokinetics of ISIS 2302, a 20-mer phosphorothioate oligodeoxynucleotide, were determined after a 2-h infusion. Doses from 0.6 to 2.0 mg/kg were studied and the peak plasma concentrations were shown to increase linearly with dose, with the 2 mg/kg dose resulting in peak plasma concentrations of intact drug of approximately 9.5 μg/ml. Clearance from plasma, however, was dose dependent, with the 2 mg/kg dose having a clearance of 1.28 ml min^{-1} kg^{-1}, while that of 0.5 mg/kg was 2.07 ml min^{-1} kg^{-1}. Essentially, no intact drug was found in urine.

Clearly, the two most recent studies differ from the initial report in several facets. Although a number of factors may explain the discrepancies, the most likely explanation is related to the evolution of assay methodology, not to a difference between compounds. Overall, the behavior of phosphorothioates in the plasma of humans appears to be similar to that in other species.

In addition to the pharmacological effects that have been observed with phosphorothioate oligonucleotides, there are a number of lines of evidence supporting the notion that these drugs enter cells in various organs. As an

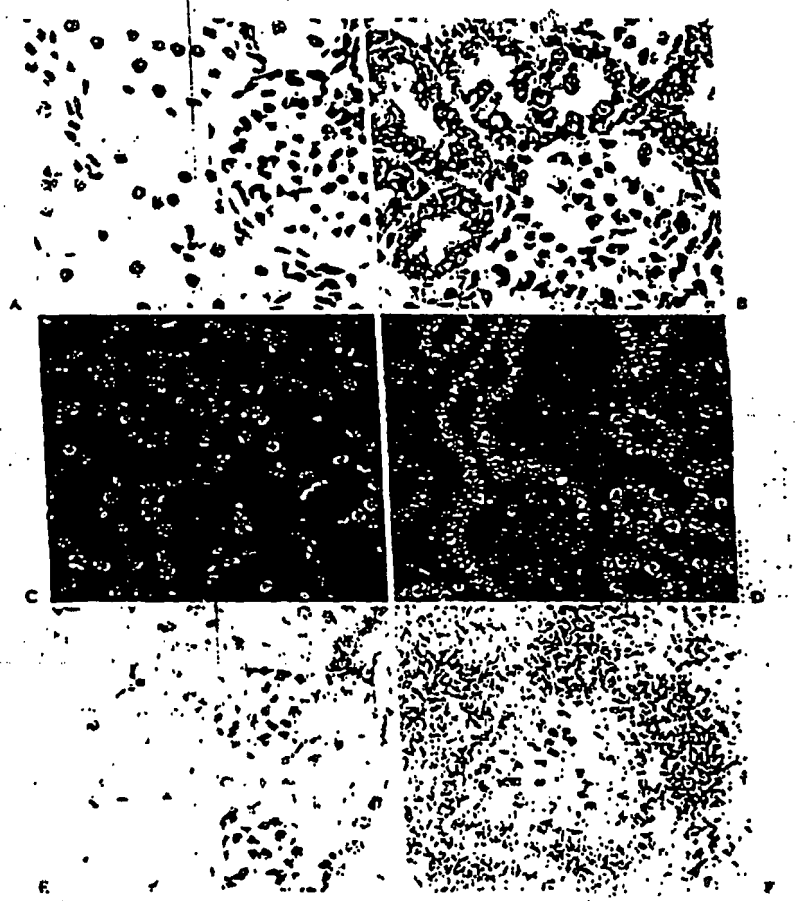

Fig. 2A–F. Autoradiographic, fluorescent, and immunohistochemical data demonstrating the intracellular location of phosphorothioate oligodeoxynucleotides in renal proximal convoluted tubular cells

example, Fig. 2 shows autoradiographic, fluorescent, and immunohistochemical data demonstrating the intracellular location of phosphorothioate oligonucleotides in renal proximal convoluted tubular cells. Similar results have been observed in liver, skin, and bone marrow in similar studies. Using radiolabeled drugs and isolated perfused rat liver cells, uptake into parenchymal and nonparenchymal cells of the liver (Takakura et al. 1996) has been reported.

We have also performed oral bioavailability experiments in rodents treated with an H_2 receptor antagonist to avoid acid-mediated depurination or precipitation. In these studies, very limited (<5%) bioavailability was observed (S. Crooke, unpublished observations). However, it seems likely that the principal limiting factor in the oral bioavailability of phosphorothioates may be degradation in the gut rather than absorption. Studies using everted rat jejunum sacs demonstrated passive transport across the intestinal epithelium (Hughes et al. 1995). Further, studies using more stable 2'-methoxy phosphorothioate oligonucleotides showed a significant increase in oral bioavailability that appeared to be associated with the improved stability of the analogs (Agrawal et al. 1995).

In summary, pharmacokinetic studies of several phosphorothioates demonstrate that they are well absorbed from parenteral sites, distribute broadly to all peripheral tissues, do not cross the blood–brain barrier and are eliminated primarily by metabolism. In short, systemic dosing should be feasible once a day or every other day. Although the similarities between oligonucleotides of different sequences are far greater than the differences, additional studies are required before determining whether there are subtle effects of sequence on the pharmacokinetic profile of this class of drugs.

V. Pharmacological Properties

1. Molecular Pharmacology

Antisense oligonucleotides are designed to bind to RNA targets via Watson-Crick hybridization. As RNA can adopt a variety of secondary structures via Watson-Crick hybridization, one useful way to think of antisense oligonucleotides is as competitive antagonists for self-complementary regions of the target RNA. Obviously, creating oligonucleotides with the highest affinity per nucleotide unit is pharmacologically important, and a comparison of the affinity of the oligonucleotide to a complementary RNA oligonucleotide is the most sensible comparison. In this context; phosphorothioate oligodeoxynucleotides are relatively competitively disadvantaged as the affinity per nucleotide unit of oligomer is less than RNA (>−2.0°C T_m per unit; Cook 1993). This results in a requirement of at least 15–17 nucleotides in order to have sufficient affinity to produce biological activity (Monia et al. 1992).

Although multiple mechanisms by which an oligonucleotide may terminate the activity of an RNA species to which it binds are possible, examples of

biological activity have been reported for only three of these mechanisms. Antisense oligonucleotides have been reported to inhibit RNA splicing, affect translation of mRNA, and induce degradation of RNA by RNase H (Crooke et al. 1991; Kulka et al. 1989; Agrawal et al. 1988). Without question, the mechanism that has resulted in the most potent compounds and is best understood is RNase H activation. To serve as a substrate for RNase H, a duplex between RNA and a "DNA-like" oligonucleotide is required. Specifically, a sugar moiety in the oligonucleotide that induces a duplex conformation equivalent to that of a DNA–RNA duplex and a charged phosphate are required (Minabelli and Crooke 1993). Thus, phosphorothioate oligodeoxynucleotides are expected to induce RNase H-mediated cleavage of the RNA when bound. As will be discussed later, many chemical approaches that enhance the affinity of an oligonucleotide for RNA result in duplexes that are no longer substrates for RNase H.

Selection of sites at which optimal antisense activity may be induced in a RNA molecule is complex, dependent on terminating mechanism and influenced by the chemical class of the oligonucleotide. Each RNA appears to display unique patterns of sites of sensitivity. Within the phosphorothioate oligodeoxynucleotide chemical class, studies in our laboratory have shown antisense activity can vary from undetectable to 100% by shifting an oligonucleotide by just a few bases in the RNA target (Chiang et al. 1991; Crooke 1992; Bennett and Crooke 1996). Although significant progress has been made in developing general rules that help define potential active sites in RNA species, to a large extent, this remains an empirical process that must be performed for each RNA target and every new chemical class of oligonucleotides.

Phosphorothioates have also been shown to have effects inconsistent with the antisense mechanism for which they were designed. Some of these effects are due to sequence or are structure specific. Others are due to nonspecific interactions with proteins. These effects are particularly prominent in in vitro tests for antiviral activity as high concentrations of cells, viruses, and oligonucleotides are often coincubated (Azad et al. 1993; Wagner et al. 1993). Human immune deficiency virus (HIV) is particularly problematic as many oligonucleotides bind to the gp120 protein (Wyatt et al. 1994). However, the potential for confusion arising from the misinterpretation of an activity as being due to an antisense mechanism when, in fact, it is due to nonantisense effects is certainly not limited to antiviral or just in vitro tests (Barrow and Lemoine 1995; Burgess et al. 1995; Hertl et al. 1995). Again, these data simply urge caution and argue for careful dose-response curves, direct analysis of target protein or RNA, and inclusion of appropriate controls before drawing conclusions concerning the mechanisms of action of oligonucleotide-based drugs. In addition to protein interactions, other factors such as overrepresented sequences of RNA and unusual structures that may be adopted by oligonucleotides, can contribute to unexpected results (Wyatt et al. 1994).

Given the variability in cellular uptake of oligonucleotides, the variability in potency as a function of binding site in an RNA target, and potential nonantisense activities of oligonucleotides, careful evaluation of dose-response curves and clear demonstration of the antisense mechanism are required before drawing conclusions from in vitro experiments. Nevertheless, numerous well-controlled studies have been reported in which antisense activity was conclusively demonstrated. As many of these studies have been reviewed previously, suffice it to say that antisense effects of phosphorothioate oligodeoxynucleotides against a variety of targets are well documented (Crooke and Lebleu 1993; Stein and Cheng 1993; S.T. Crooke 1992, 1993, 1995b; Nagel et al. 1993).

2. In Vivo Pharmacological Activities

A relatively large number of reports of in vivo activities of phosphorothioate oligonucleotides have now appeared documenting activities both after local and systemic administration (Table 1; S.T. Crooke 1995b). However, for only a few of these reports have sufficient studies been performed to draw relatively firm conclusions concerning the mechanism of action. Consequently, I will review in some detail only a few reports that provide sufficient data to support a relatively firm conclusion with regard to mechanism of action. Local effects have been reported for phosphorothioate and methylphosphonate oligonucleotides. A phosphorothioate oligonucleotides designed to inhibit *c-myb* production and applied locally was shown to inhibit intimal accumulation in the rat carotid artery (Simons et al. 1992). In this study, a Northern blot analysis showed a significant reduction in *c-myb* RNA in animals treated with the antisense compound, but no effect when treated with a control oligonucleotide. In a recent study, it was suggested that the effects of the oligonucleotide were due to a nonantisense mechanism (Burgess et al. 1995). However, only one dose level was studied, so much remains to be done before definitive conclusions are possible. Similar effects were reported for phosphorothioate oligodeoxynucleotides designed to inhibit cyclin-dependent kinases (CDC-2 and CDK-2). Again, the antisense oligonucleotide inhibited intimal thickening and cyclin-dependent kinase activity, while a control oligonucleotide had no effect (Ase et al. 1994). Additionally, local administration of a phosphorothioate oligonucleotide designed to inhibit N-*myc* resulted in reduction in N-*myc* expression and slower growth of a subcutaneously transplanted human tumor in nude mice (Whitesell et al. 1991).

Antisense oligonucleotides administered intraventricularly have been reported to induce a variety of effects in the central nervous system. Intraventricular injection of antisense oligonucleotides to neuropeptide Y-Y1 receptor mRNA reduced the density of the receptors and resulted in behavioral signs of anxiety (Wahlestedt et al. 1993). Similarly, an antisense oligonucleotide designed to bind to *N*-methyl-D-aspartate (NMDA)-R1 receptor channel RNA inhibited the synthesis of these channels and reduced the volume of focal

Table 1. Reported activity of antisense oligonucleotides in animal models

Target	Route	Species	Reference
Cardiovascular models			
c-myb	Topically	Rat	(Simons et al. 1992)
cdc2 kinase	Topically	Rat	(Morishita et al. 1993)
PCNA	Topically	Rat	(Morishita et al. 1993)
cdc2 kinase	Topically	Rat	(Ase et al. 1994)
CDK2	Topically	Rat	(Ase et al. 1994)
Cyclin B,	Topically	Rat	(Morishita et al. 1994)
PCNA	Topically	Rat	(Simons et al. 1994)
Angiotensin type 1 receptor	Intracerebral	Rat	(Gyulkó et al. 1993)
Angiotensinogen	Intracerebral	Rat	(Phillips et al. 1994)
c-fos	Intracerebral	Rat	(Suzuki et al. 1994)
Inflammatory models			
Type I IL-1 receptor	Intradermal	Mouse	(Bunch and Mahan 1991)
ICAM-1	Intravenous	Mouse	(Stepkowski et al. 1994)
ICAM-1	Intravenous	Mouse	(Kumagaia et al. 1996)
ICAM-1	Intravenous	Mouse	(Kaye et al. 1995)
ICAM-1	Intravenous	Mouse	(Stepkowski et al. 1995)
ICAM-1	Intravenous	Mouse	(Bennett et al. 1997)
Adenosine type 1 receptor	Aerosol	Rabbit	(Nyce and Metzger 1997)
Cancer models			
N-*myc*	Subcutaneous	Mouse	(Whitesell et al. 1991)
NF-kB p65	Intraperitoneal	Mouse	(Kitajima et al. 1992)
c-myb	Subcutaneous	Mouse	(Ratajczak et al. 1992)
p120 nucleolar antigen	Intraperitoneal	Mouse	(Pichiarry et al. 1993)
NK-kB p65	Subcutaneous	Mouse	(Higgins et al. 1993)
Protein kinase C-α	Intraperitoneal	Mouse	(Dean and McKay 1994)
c-myb	Subcutaneous	Mouse	(Hijiya et al. 1994)
Ha-*ras*	Intratumor	Mouse	(Schwab et al. 1994)
BCR-ABL	Intravenous	Mouse	(Szczylik et al. 1994)
PTHrP	Intraventricular	Rat	(Akino et al. 1995)
c-raf kinase	Intravenous	Mouse	(Monia et al. 1993)
Protein kinase C-α	Intravenous	Mouse	(Dean et al. 1996)
Protein kinase C-α	Intravenous	Mouse	(Yazaki et al. 1994)
Neurological models			
c-fos	Intracerebral	Rat	(Chiasson et al. 1992)
SNAP-25	Intracerebral	Chicken	(Osen-Sand et al. 1993)
Kinesin heavy chain	Intravitreal	Rabbit	(Ahmadiunga et al. 1993)
Arginine vasopressin	Intracerebral	Rat	(Flanagan et al. 1993)
c-fos	Intracerebral	Rat	(Hanlo et al. 1993)
Progesterone receptor	Intracerebral	Rat	(Pollio et al. 1993)
Dopamine type 2 receptor	Intracerebral	Rat	(Zhang and Creese 1993)
Y-Y1 receptor	Intracerebral	Rat	(Wahlestedt et al. 1993)
Neuropeptide Y	Intracerebral	Rat	(Akabayashi et al. 1994)
k-opioid receptor	Intracerebral	Rat	(Adams et al. 1994)
IGF-1	Intracerebral	Rat	(Castro-Alamancos and Torres-Aleman 1994)
c-fos	Intraspinal	Rat	(Gillardon et al. 1994)
c-fos	Intracerebral	Rat	(Hooper et al. 1994)
c-fos	Intraspinal	Rat	(Woodbury et al. 1994)
NMDA receptor	Intracerebral	Rat	(Kindy 1994)
CREB	Intracerebral	Rat	(Konradi et al. 1994)

Table 1. *Continued*

Target	Route	Species	Reference
Delta-opioid receptor	Intracerebral	Mice	(Lai et al. 1994)
Progesterone receptor	Intracerebral	Rat	(Mani et al. 1994)
GAD65	Intracerebral	Rat	(McCarthy et al. 1994)
GAD67	Intracerebral	Rat	(McCarthy et al. 1994)
AT$_2$-angiotensin receptor	Intracerebral	Rat	(Sakai et al. 1995)
Tryptophan hydroxylase	Intracerebral	Mouse	(McCarthy et al. 1995)
AT1-angiotensin receptor	Intracerebral	Rat	(Ambühl et al. 1995)
CRH$_1$-corticotropin-releasing	Intracerebral	Rat	(Liebsch et al. 1995)
Hormone receptor			
• Opiod receptor	Intracerebral	Rat	(Chia et al. 1995)
• Opiod receptor	Intracerebral	Mouse	(Mizoguchi et al. 1995)
Oxytocin	Intracerebral	Rat	(Mahieu et al. 1995)
Oxytocin	Intracerebral	Rat	(Neumann et al. 1994)
Substance P receptor	Intracerebral	Rat	(Ono et al. 1994)
Tyrosine hydroxylase	Intracerebral	Rat	(Skuterud et al. 1994)
c-jun	Intracerebral	Rat	(Tuschinevez et al. 1994)
Dopamine type 1 receptor	Intracerebral	Mouse	(Zhang et al. 1994)
Dopamine type 2 receptor	Intracerebral	Mouse	(Zhou et al. 1994)
Dopamine type 2 receptor	Intracerebral	Mouse	(Weiss et al. 1993)
Dopamine type 3 receptor	Intracerebral	Mouse	(Qin et al. 1995)
Viral models			
HSV-1		Mouse	(Kulka et al. 1989)
Tick-born encephalitis		Mouse	(Vlasov 1989)
Duck hepatitis virus	Intravenous	Duck	(Offensperger et al. 1993)

ischemia produced by occlusion of the middle cerebral artery in rats (Wahlestedt et al. 1993).

In a series of well-controlled studies, antisense oligonucleotides administered intraventricularly selectively inhibited dopamine type-2 receptor expression, dopamine type-2 receptor RNA levels, and behavioral effects in animals with chemical lesions. Controls included randomized oligonucleotides and the observation that no effects were observed on dopamine type-1 receptor or RNA levels (Weiss et al. 1993; Zhou et al. 1994; Qin et al. 1995). This laboratory also reported the selective reduction of dopamine type-1 receptor and RNA levels with the appropriate oligonucleotide (Zhang et al. 1994).

Similar observations were reported in studies on angiotensin type 1 (AT-1) receptors and tryptophan hydroxylase. In studies in rats, direct observations of AT-1 and AT-2 receptor densities in various sites in the brain after administration of different doses of phosphorothioate antisense, sense, and scrambled oligonucleotides were reported (Ambühl et al. 1995). Again, in rats, intraventricular administration of an antisense phosphorothioate oligonucleotide resulted in a decrease in tryptophan hydroxylase levels in the brain, while a scrambled control did not (McCarthy et al. 1995).

Injection of antisense oligonucleotides to synaptosomal-associated protein-25 into the vitreous body of rat embryos reduced the expression of the

protein and inhibited neurite elongation by rat cortical neurons (Osen-Sand et al. 1993).

Aerosol administration to rabbits of an antisense phosphorothioate oligodeoxynucleotide designed to inhibit the production of antisense A$_1$ receptor has been reported to reduce receptor numbers in the airway smooth muscle and to inhibit adenosine, house dust mite allergen, and histamine-induced bronchoconstriction (Nyce and Metzger 1997). Neither control nor an oligonucleotide complementary to bradykinin B$_2$ receptors reduced adenosine A$_1$ receptors' density, although the oligonucleotides complementary to bradykinin B$_2$ receptor mRNA reduced the density of these receptors.

In addition to local and regional effects of antisense oligonucleotides, a growing number of well-controlled studies have demonstrated systemic effects of phosphorothioate oligodeoxynucleotides. Expression of interleukin-[illegible] mice was inhibited by systemic administration of antisense oligonucleotides (Burch and Mahan 1991). Oligonucleotides to the NF-kB p65 subunit administered intraperitoneally at 40 mg/kg every 3 days slowed tumor growth in mice transgenic for the human T-cell leukemia viruses (Kitajima et al. 1992). Similar results with other antisense oligonucleotides were shown in another tumor model after either prolonged subcutaneous infusion or intermittent subcutaneous injection (Higgins et al. 1993).

Several recent reports further extend the studies of phosphorothioate oligonucleotides as antitumor agents in mice. In one study, a phosphorothioate oligonucleotide directed to inhibition of the *bcr-abl* junction was administered at a dose of 1 mg/day for 9 days intravenously to immunodeficient mice injected with human leukemic cells. The drug was shown to inhibit the development of leukemic colonies in the mice and to selectively reduce *bcr-abl* RNA levels in peripheral blood lymphocytes, spleen, bone marrow, liver, lungs, and brain (Skorski et al. 1994). However, it is possible that effects on the RNA levels were secondary to effects on the growth of various cell types. In the second study, a phosphorothioate oligonucleotide antisense to the protooncogene *myb*, inhibited the growth of human melanoma in mice. Again, *myb* mRNA levels appeared to be selectively reduced (Hurvitz et al. 1994).

A number of studies from our laboratories that directly examined target RNA levels, target protein levels, and pharmacological effects using a wide range of control oligonucleotides and examination of the effects on closely related isotypes have been completed. Single and chronic daily administration of a phosphorothioate oligonucleotide designed to inhibit mouse protein kinase C-α (PKC-α), selectively inhibited expression of PKC-α RNA in mouse liver without effects on any other isotype. The effects lasted at least 24 h after a dose and a clear dose-response curve was observed, with a dose of 10–15 mg/kg intraperitoneally reducing PKC-α RNA levels in liver by 50% 24 h after dose (Dean and McKay 1994).

A phosphorothioate oligonucleotide designed to inhibit human PKC-α expression selectively inhibited expression of PKC-α RNA [illegible]

protein in human tumor cell lines implanted subcutaneously in nude mice after intravenous administration (Dean et al. 1996). In these studies, effects on RNA and protein levels were highly specific and observed at doses lower than 6 mg/kg per day and antitumor effects were detected at doses as low as 0.6 mg/kg per day. A large number of control oligonucleotides failed to show activity.

In a similar series of studies, Monia et al. demonstrated highly specific loss of human *c-raf* kinase RNA in human tumor xenografts and antitumor activity that correlated with the loss of RNA. Moreover, a series of control oligonucleotides with 1–7 mismatches showed decreasing potency in vitro and precisely the same rank order potencies in vivo (Monia et al. 1995, 1996).

Finally, a single injection of a phosphorothioate oligonucleotide designed to inhibit c-AMP-dependent protein kinase type 1 was reported to selectively reduce RNA and protein levels in human tumor xenografts and to reduce tumor growth (Nesterova and Cho-Chung 1995).

Thus, there is a growing body of evidence that phosphorothioate oligonucleotides can induce potent systemic and local effects in vivo. More importantly, there are now a number of studies with sufficient controls and direct observation of target RNA and protein levels to suggest highly specific effects that are difficult to explain via any mechanism other than antisense. As would be expected, the potency of these effects varies depending on the target, the organ, and the endpoint measured as well as the route of administration and the time point after administration at which the effect is measured.

In conclusion, although it is of obvious importance to interpret in vivo activity data cautiously, and it is clearly necessary to include a range of controls and to evaluate effects on target RNA and protein levels and control RNA and protein levels directly, it is difficult to argue with the conclusion today that some effects have been observed in animals that are most likely primarily due to an antisense mechanism.

Additionally, in studies on patients with cytomegalovirus-induced retinitis, local injections of ISIS 2922 have resulted in impressive efficacy, though it is obviously impossible to prove the mechanism of action is antisense in these studies (Hutcherson et al. 1995). More recently, ISIS 2302, an ICAM-1 inhibitor, was reported to result in statistically significant reductions in steroid doses and prolonged remissions in a small group of steroid-dependent patients with Crohn's Disease. As this study was randomized, double-blinded, and included serial colonoscopies, it may be considered the first study in humans to demonstrate the therapeutic activity of an antisense drug after systemic administration (Yacyshyn et al. 1997).

VI. Toxicological Properties

1. In Vitro

In our laboratory, we have evaluated the toxicities of scores of phosphorothioate oligodeoxynucleotides in a significant number of cell lines in

tissue culture. As a general rule, no significant cytotoxicity is induced at concentrations below 100 μM oligonucleotide. Additionally, with a few exceptions, no significant effect on macromolecular synthesis is observed at concentrations below 100 μM (R.M. Crooke 1993a,b).

Polynucleotides and other polyanions have been shown to cause release of cytokines (Colby 1971). Also, bacterial DNA species have been reported to be mitogenic for lymphocytes in vitro (Messina et al. 1991). Furthermore, oligodeoxynucleotides (30–45 nucleotides in length) were reported to induce interferons and enhance natural killer cell activity (Kuramoto et al. 1992). In the latter study, the oligonucleotides that displayed natural killer cell (NK)-stimulating activity contained specific palindromic sequences and tended to be guanosine-rich. Collectively, these observations indicate that nucleic acids may have broad immunostimulatory activity.

It has been shown that phosphorothioate oligonucleotides stimulate B-lymphocyte proliferation in a mouse splenocyte preparation (analogous to bacterial DNA), and the response may underlie the observations of lymphoid hyperplasia in the spleen and lymph nodes of rodents caused by repeated administration of these compounds (see below; Pisetsky and Reich 1994). We also have evidence of enhanced cytokine release by immunocompetent cells when exposed to phosphorothioates in vitro (R.M. Crooke et al. 1996). In this study, both human keratinocytes and an in vitro model of human skin released interleukin-1α when treated with 250 μM–1 mm of phosphorothioate oligonucleotides. The effects seemed to be dependent on the phosphorothioate backbone and independent of sequence or 2′ modification. In a study in which murine B lymphocytes were treated with phosphodiester oligonucleotides, B-cell activation was induced by oligonucleotides with unmethylated CpG dinucleotides (Krieg et al. 1995). This has been extrapolated to suggest that the CpG motif may be required for immune stimulation of oligonucleotide analogs such as phosphorothioates. This clearly is not the case with regard to release of IL-1α from keratinocytes (R.M. Crooke et al. 1996). Nor is it the case with regard to in vivo immune stimulation (see below).

2. Genotoxicity

As with any new chemical class of therapeutic agents, concerns about genotoxicity cannot be dismissed as little in vitro testing has been performed and no data from long-term studies of oligonucleotides are available. Clearly, given the limitations in our understanding about the basic mechanisms that might be involved, empirical data must be generated. We have performed mutagenicity studies on two phosphorothioate oligonucleotides, ISIS 2105 and ISIS 2922, and found them to be nonmutagenic at all concentrations studied (S.T. Crooke et al. 1994).

Two mechanisms of genotoxicity that may be unique to oligonucleotides have been considered. One possibility is that an oligonucleotide analog could be integrated into the genome and produce mutagenic events. Although inte-

gration of an oligonucleotide into the genome is conceivable, it is likely to be extremely rare. For most viruses, viral DNA integration is itself a rare event and, of course, viruses have evolved specialized enzyme-mediated mechanisms to achieve integration. Moreover, preliminary studies in our laboratory have shown that phosphorothioate oligodeoxynucleotides are generally poor substrates for DNA polymerases, and it is unlikely that enzymes such as integrases, gyrases, and topoisomerases (that have obligate DNA cleavage as intermediate steps in their enzymatic processes) will accept these compounds as substrates. Consequently, it would seem that the risk of genotoxicity due to genomic integration is no greater and probably less than that of other potential mechanisms, for example, alteration of the activity of growth factors, cytokine release, nonspecific effects on membranes that might trigger arachidonic acid release, or inappropriate intracellular signaling. Presumably, new analogs that deviate more significantly from natural DNA would be even less likely to be integrated.

A second concern that has been raised about possible genotoxicity is the risk that oligonucleotides might be degraded to toxic or carcinogenic metabolites. However, metabolism of phosphorothioate oligodeoxynucleotides by base excision would release normal bases, which presumably would be nongenotoxic. Similarly, oxidation of the phosphorothioate backbone to the natural phosphodiester structure would also yield nonmutagenic (and probably nontoxic) metabolites. Finally, it is possible that phosphorothioate bonds could be hydrolyzed slowly, releasing nucleoside phosphorothioates that presumably would be rapidly oxidized to natural (nontoxic) nucleoside phosphates. However, oligonucleotides with modified bases and/or backbones may pose different risks.

3. In Vivo

The acute LD_{50} in mice of all phosphorothioate oligonucleotides tested to date is in excess of 500 mg/kg (D. Kornbrust, unpublished observations). In rodents, we have had the opportunity to evaluate the acute and chronic toxicities of multiple phosphorothioate oligonucleotides administered by multiple routes (Henry et al. 1997c,d). The consistent dose limiting toxicity was immune stimulation manifested by lymphoid hyperplasia, splenomegaly, and a multiorgan monocellular infiltrate. These effects occurred only with chronic dosing at doses greater than 20 mg/kg and were dose dependent. The liver and kidney were the organs most prominently affected by monocellular infiltrates. All of these effects appeared to be reversible and chronic intradermal administration appeared to be the most toxic route, probably because of high local concentrations of the drugs resulting in local cytokine release and initiation of a cytokine cascade. There were no obvious effects of sequence. At doses of 100 mg/kg and greater, minor increases in liver enzyme levels and mild thrombocytopenia were also observed.

In monkeys, however, the toxicological profile of phosphorothioate oligonucleotides is quite different. The most prominent dose-limiting side effect is

sporadic reductions in blood pressure associated with bradycardia. When these events are observed, they are often associated with activation of C5 complement and they are dose related and peak plasma concentration related. This appears to be related to the activation of the alternative pathway (Henry et al. 1997a). All phosphorothioate oligonucleotides tested to date appear to induce these effects, though there may be slight variations in potency as a function of sequence and/or length (Cornish et al. 1993; Galbraith et al. 1994; Henry et al. 1997c,d).

A second prominent toxicologic effect in the monkey is the prolongation of activated partial thromboplastin time. At higher doses, evidence of clotting abnormalities is observed. Again, these effects are dose and peak plasma concentration dependent (Galbraith et al. 1994; Henry et al. 1997b). Although no evidence of sequence dependence has been observed, there appears to be a linear correlation between number of phosphorothioate linkages and potency between 18–25 nucleotides (P. Nicklin, unpublished observations). The mechanisms responsible for these effects are likely very complex, but preliminary data suggest that direct interactions with thrombin may be at least partially responsible for the effects observed (Henry et al. 1997b).

In man, the toxicological profile again differs a bit. When ISIS 2922 is administered intravitreally to patients with cytomegalovirus retinitis, the most common adverse event is anterior chamber inflammation, which is easily managed with steroids. A relatively rare and dose-related adverse event is morphological changes in the retina associated with loss in peripheral vision (Hutcherson et al. 1995).

ISIS 2105, a 20-mer phosphorothioate designed to inhibit the replication of human papilloma viruses that cause genital warts, is administered intradermally at doses as high as 3 mg/wart weekly for 3 weeks; essentially, no toxicities have been observed, including remarkably, a complete absence of local inflammation (L. Grillone, unpublished results).

Every other day administration of 2-h intravenous infusions of ISIS 2302 at doses as high as 2 mg/kg resulted in no significant toxicities, including no evidence of immune stimulation and no hypotension. A slight subclinical increase in activated partial thromboplastin time was observed at the 2 mg/kg dose (Glover et al. 1996).

VII. Therapeutic Index

In Fig. 3, an attempt to put the toxicities and their dose-response relationships in a therapeutic context is shown. This is particularly important as considerable confusion has arisen concerning the potential utility of phosphorothioate oligonucleotides for selected therapeutic purposes, deriving from unsophisticated interpretation of toxicological data. As can readily be seen, the immune stimulation induced by these compounds appears to be particularly prominent in rodents and unlikely to be dose limiting in man. Nor have we, to date, observed hypotensive events in humans. Thus, this toxicity

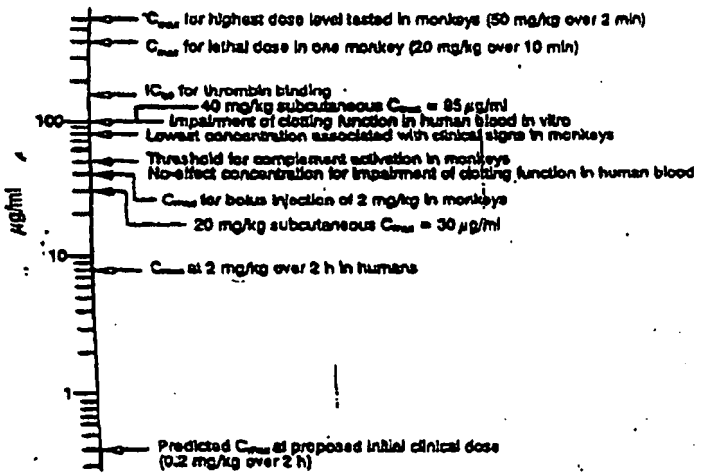

Fig. 3. Plasma concentrations of ISIS 2302 at which various activities are observed. These concentrations are determined by extracting plasma and analyzing by capillary gel electrophoresis and represent intact ISIS 2302

appears to occur at lower doses in monkeys than man and certainly is not dose limiting in man.

Based on our experience to date, we believe that the dose-limiting toxicity in man will be clotting abnormalities, and this will be associated with peak plasma concentrations well in excess of 10 μg/ml. In animals, pharmacological activities have been observed with I.V. bolus doses from 0.006 to 10–15 mg/kg depending on the target, the endpoint, the organ studied and the time after a dose when the effect is measured. Thus, it would appear that phosphorothioate oligonucleotides have a therapeutic index that supports their evaluation for a number of therapeutic indications.

VIII. Conclusions

Phosphorothioate oligonucleotides have perhaps outperformed many expectations. They display attractive parenteral pharmacokinetic properties. They have produced potent systemic effects in a number of animal models and, in many experiments, the antisense mechanism has been directly demonstrated as the hoped-for selectivity. Further, these compounds appear to display satisfactory therapeutic indices for many indications.

Table 2. Phosphorothioate oligonucleotides

Limits
Pharmacodynamic
• Low affinity per nucleotide unit
• Inhibition of RNase H at high concentrations
Pharmacokinetic
• Limited bioavailability
• Limited blood–brain barrier penetration
• Dose-dependent pharmacokinetics
• Possible drug–drug interactions
Toxicologic
• Release of cytokines
• Complement associated effects on blood pressure?
• Clotting effects

Nevertheless, phosphorothioates clearly have significant limits (Table 2). Pharmacodynamically, they have relatively low affinity per nucleotide unit. This means that longer oligonucleotides are required for biological activity and that invasion of many RNA structures may not be possible. At higher concentrations, these compounds inhibit RNase H as well. Thus, the higher end of the pharmacologic dose response curve is lost. Pharmacokinetically, phosphorothioates do not cross the blood–brain barrier, are not significantly orally bioavailable, and may display dose-dependent pharmacokinetics. Toxicologically, clearly the release of cytokines, activation of complement, and interference with clotting will pose dose limits if they are encountered in the clinic.

As several clinical trials are in progress with phosphorothioates and others will be initiated shortly, we shall soon have more definitive information about the activities, toxicities, and value of this class of antisense drugs in human beings.

E. The Medicinal Chemistry of Oligonucleotides

1. Introduction

The core of any rational drug discovery program is medicinal chemistry. Although the synthesis of modified nucleic acids has been a subject of interest for some time, the intense focus on the medicinal chemistry of oligonucleotides arguably predates this chapter by no more than 5 years. Consequently, the scope of medicinal chemistry has recently expanded enormously, but the biological data to support conclusions about synthetic strategies are only beginning to emerge.

Modifications in the base, sugar, and phosphate moieties of oligonucleotides have been reported. The subjects of medicinal chemical programs in-

clude approaches to create enhanced affinity and more selective affinity for RNA or duplex structures, the ability to cleave nucleic acid targets, enhanced nuclease stability, cellular uptake and distribution, and in vivo tissue distribution, metabolism and clearance.

II. Heterocycle Modifications

1. Pyrimidine Modifications

A relatively large number of modified pyrimidines have been synthesized and are now incorporated into oligonucleotides and evaluated. The principle sites of modification are C-2, C-4, C-5, and C-6. These and other nucleoside analogs have recently been thoroughly reviewed (Sanghvi 1993). Consequently, a very brief summary of the analogs that displayed interesting properties is incorporated here.

Inasmuch as the C-2 position is involved in Watson-Crick hybridization, C-2 modified pyrimidine containing oligonucleotides have shown unattractive hybridization properties. An oligonucleotide containing 2-thiothymidine was found t hybridize well to DNA and, in fact, even better to RNA (ΔT_m 1.5°C modificati n) (E. Swayze et al., unpublished results).

In contrast, several modifications in the 4 position that have interesting properties have been reported. 4-Thiopyrimidines have been incorporated into oligonucleotides with no significant negative effect on hybridization (Nikiforov and Connolly 1991). A bicyclic and an N4-methoxy analog of cytosine were shown to hybridize with both purine bases in DNA with T_m's approximately equal to natural base pairs (Lin and Brown 1989). Additionally, a fluorescent base has been incorporated into oligonucleotides and shown to enhance DNA–DNA duplex stability (Inoue et al. 1985).

A large number of modifications at the C-5 position have also been reported, including halogenated nucleosides. Although the stability of duplexes may be enhanced by incorporating 5-halogenated nucleosides, the occasional mispairing with G, and the potential that the oligonucleotide might degrade and release toxic nucleosides analogs cause concern (Sanghvi 1993).

Furthermore, oligonucleotides containing 5-propynylpyrimidine modifications have been shown to enhance the duplex stability (ΔT_m 1.6°C/modification) and support the RNase H activity. The 5-heteroarylpyrimidines were also shown to influence the stability of duplexes (Wagner et al. 1993; Gutierrez et al. 1994). A more dramatic influence was reported for the tricyclic 2'-deoxycytidine analogs, exhibiting an enhancement of 2°C–5°C/modification, depending on the positioning of the modified bases (Lin et al. 1995). It is believed that the enhanced binding properties of these analogs is due to extended stacking and increased hydrophobic interactions.

In general, as expected, modifications in the C-6 position of pyrimidines are highly duplex destabilizing (Sanghvi et al. 1993). Oligonucleotides containing 6-aza pyrimidines have been shown to reduce T_m by 4°C–2°C per modification, but to enhance the nuclease stability of oligonucleotides and to support RNase H-induced degradation of RNA targets (Sanghvi 1993).

2. Purine Modifications

Although numerous purine analogs have been synthesized, when incorporated into oligonucleotides, they usually have resulted in destabilization of duplexes. However, there are a few exceptions, where a purine modification had a stabilizing effect. A brief summary of some of these analogs is discussed below.

Generally, N1 modifications of purine moiety has resulted in destabilization of the duplex (Manoharan 1993). Similarly, C2 modifications have usually resulted in destabilization. However, 2-6-diaminopurine has been reported to enhance hybridization by approximately 1°C per modification when paired with T (Sproat et al. 1991). Of the 3-position substituted bases reported to date, only the 3-deaza adenosine analog has been shown t have a effective on hybridization.

Modifications at the C-6 and C-7 positions have likewise resulted in only a few interesting bases from the point of view of hybridization. Inosine has been shown to have little effect on duplex stability, but because it can pair and stack with all four normal DNA bases, it behaves as a universal base and creates an ambiguous position in an oligonucleotide (Martin et al. 1985). Incorporation of 7-deaza inosine into oligonucleotides was destabilizing and this was considered to be due to its relatively hydrophobic nature (Santalucia et al. 1991). 7-Deaza guanine was similarly destabilizing, but when 8-aza-7-deaza guanine was incorporated into oligonucleotides, it enhanced hybridizations (Seela et al. 1989). Thus, on occasion, introduction of more than one modification in a nucleobase may compensate for destabilizing effects of some modifications. Interestingly, 7-iodo 7-deazaguanine residue was recently incorporated into oligonucleotides and shown to enhance the binding affinity dramatically (ΔT_m 10.0°C/modification compared to 7-deazaguanine; Seela et al. 1995). The increase in T_m value was attributed to (a) the hydrophobic nature of the modification, (b) increased stacking interaction, and (c) favorable pKa of the base.

In contrast, some C8 substituted bases have yielded improved nuclease resistance when incorporated in oligonucleotides, but seem to be somewhat destabilizing (Sanghvi 1993).

3. Oligonucleotide Conjugates

Although conjugation of various functionalities to oligonucleotides has been reported to achieve a number of important objectives, the data supporting some of the claims are limited and generalizations are not possible based on the data presently available.

a) Nuclease Stability

Numerous 3' modifications have been reported to enhance the stability of oligonucleotides in serum (Manoharan 1993). Both neutral and charged substituents have been reported to stabilize oligonucleotides in serum and, as a general rule, the stability of a conjugated oligonucleotide tends to be greater as bulkier substituents are added. Inasmuch as the principle nuclease in serum is a 3' exonuclease, it is not surprising that 5' modifications have resulted in significantly less stabilization. Internal modifications of base, sugar, and backbone have also been reported to enhance nuclease stability at or near the modified nucleoside (Manoharan 1993). In a recent study, thiono triester (adamantyl, cholesteryl and others) modified oligonucleotides have shown improved nuclease stability, cellular association, and binding affinity (Zhang et al. 1995).

The demonstration that modifications may induce nuclease stability sufficient to enhance activity in cells in tissue culture and in animals has proven to be much more complicated because of the presence of 5' exonucleases and endonucleases. In our laboratory, 3' modifications and internal point modifications have not provided sufficient nuclease stability to demonstrate pharmacological activity in cells (Hoke et al. 1991). In fact, even a 5 nucleotide long phosphodiester gap in the middle of a phosphorothioate oligonucleotide resulted in sufficient loss of nuclease resistance to cause complete loss of pharmacological activity (Monia et al. 1992).

In mice, neither a 5'-cholesterol nor 5'-C18 amine conjugate altered the metabolic rate of a phosphorothioate oligodeoxynucleotide in liver, kidney, or plasma (S.T. Crooke et al. 1996). Furthermore, blocking the 3' and 5' termini of a phosphodiester oligonucleotide did not markedly enhance the nuclease stability of the parent compound in mice (Sands et al. 1995). However, 3' modification of a phosphorothioate oligonucleotide was reported to enhance its stability in mice relative to the parent phosphorothioate (Temsamani et al. 1993). Moreover, a phosphorothioate oligonucleotide with a 3'-hairpin loop was reported to be more stable in rats than its parent (Zhang et al. 1995). Thus, 3' modifications may enhance the stability of the relatively stable phosphorothioates sufficiently to be of value.

b) Enhanced Cellular Uptake

Although oligonucleotides have been shown to be taken up by a number of cell lines in tissue culture, with perhaps the most compelling data relating to phosphorothioate oligonucleotides, a clear objective has been to improve cellular uptake of oligonucleotides (R.M. Crooke 1991; S.T. Crooke et al. 1994). Inasmuch as the mechanisms of cellular uptake of oligonucleotides are still very poorly understood, the medicinal chemistry approaches have been largely empirical and based on many unproven assumptions.

Because phosphodiester and phosphorothioate oligonucleotides are water soluble, the conjugation of lipophilic substituents to enhance membrane permeability has been a subject of considerable interest. Unfortunately, studies in this area have not been systematic and, at present, there is precious little information about the changes in physicochemical properties of oligonucleotides actually effected by specific lipid conjugates. Phospholipids, cholesterol and cholesterol derivatives, cholic acid, and simple alkyl chains have been conjugated to oligonucleotides at various sites in the oligonucleotide. The effects of these modifications on cellular uptake have been assessed using fluorescent, or radiolabeled, oligonucleotides or by measuring pharmacological activities. From the perspective of medicinal chemistry, very few systematic studies have been performed. The activities of short alkyl chains, adamantine, daunomycin, fluorescein, cholesterol, and porphyrin conjugated oligonucleotides were compared in one study (Boutorine et al. 1991). A cholesterol modification was reported to be more effective at enhancing uptake than the other substituents. It also seems likely that the effects of various conjugates on cellular uptake may be affected by the cell type and target studied. For example, we have studied cholic acid conjugates of phosphorothioate deoxyoligonucleotides or phosphorothioate 2'-methoxy oligonucleotides and observed enhanced activity against HIV and no effect on the activity of ICAM-directed oligonucleotides.

Additionally, polycationic substitutions and various groups designed to bind to cellular carrier systems have been synthesized. Although many compounds have been synthesized, the data reported to date are insufficient to draw firm conclusions about the value of such approaches or structure activity relationships (Manoharan 1993).

c) RNA Cleaving Groups

Oligonucleotide conjugates were recently reported to act as artificial ribonucleases, albeit with low efficiencies (De Mesmaeker et al. 1995). Conjugation of chemically reactive groups such as alkylating agents, photoinduced azides, porphyrin, and psoralene have been utilized extensively to effect a cross-linking of oligonucleotide and the target RNA. In principle, this treatment may lead to translation arrest. In addition, lanthanides and complexes thereof have been reported to cleave RNA via a hydrolytic pathway. Recently, a novel europium complex was covalently linked to an oligonucleotide and shown to cleave 88% of the complementary RNA at physiological pH (Hall et al. 1994).

d) In Vivo Effects

To date, relatively few studies have been reported in vivo. The properties of a 5'-cholesterol and 5'-C-18 amine conjugates of a 20-mer phosphorothioate oligodeoxynucleotide have been determined in mice. Both compounds increased the fraction of an I.V. bolus dose found in the liver. The cholesterol conjugate, in fact, resulted in more than 80% of the dose accumulating in the liver. Neither conjugate enhanced stability in plasma, liver, or kidney (S.T.

Crooke et al. 1996). Interestingly, the only significant change in the toxicity profile was a slight increase in effects on serum transaminases and histopathological changes indicative of slight liver toxicity associated with the cholesterol conjugate (Henry et al. 1997e). A 5'-cholesterol phosphorothioate conjugate was also recently reported to have a longer elimination half-life, to be more potent, and to induce greater liver toxicity in rats (Desjardins et al. 1995).

4. Sugar Modifications

The focus of second-generation oligonucleotide modifications has centered on the sugar moiety. In oligonucleotides, pentofuranose sugar ring occupies a central connecting manifold that also positions the nucleobases for effective stacking. Recently, a symposium series volume has been published on the carbohydrate modifications in antisense research that covers this topic in great detail (Sanghvi and Cook 1994). Therefore, the content of the following discussion is restricted to a summary of the main events in this area.

A growing number of oligonucleotides in which the pentofuranose ring is modified or replaced have been reported (Breslauer et al. 1986). Uniform modifications at the 2' position have been shown to enhance hybridization to RNA, and in some cases, to enhance nuclease resistance (Breslauer et al. 1986). Chimeric oligonucleotides containing 2'-deoxyoligonucleotide gaps with 2'-modified wings have been shown to be more potent than parent molecules (Monia et al. 1993).

Other sugar modifications include α-oligonucleotides, carbocyclic oligonucleotides and hexapyranosyl oligonucleotides (Breslauer et al. 1986). Of these, α-oligonucleotides have been most extensively studied. They hybridize in parallel fashion to single-stranded DNA and RNA and are nuclease resistant. However, they have been reported to be oligonucleotides designed to inhibit Ha-*ras* expression. All these oligonucleotides support RNase H and, as can be seen, a direct correlation between affinity and potency exists.

A growing number of oligonucleotides in which the C-2' position of the sugar ring is modified have been reported (Manoharan 1993; De Mesmaeker et al. 1995). These modifications include lipophilic alkyl groups, intercalators, amphipathic amino-alkyl tethers, positively charged polyamines, highly electronegative fluoro or fluoro alkyl moities, and sterically bulky methylthio derivatives. The beneficial effects of a C-2' substitution on the antisense oligonucleotide cellular uptake, nuclease resistance, and binding affinity have been well documented in the literature. In addition, excellent review articles have appeared in the last few years on the synthesis and properties of C-2'-modified oligonucleotides (De Mesmaeker et al. 1995; Lamond and Sproat 1993; Sproat and Lamond 1993; Parmentier et al. 1994).

Other modifications of the sugar moiety have also been studied, including other sites as well as more substantial modifications. However, much less is known about the antisense effects of these modifications (S.T. Crooke 1995b).

2'-Methoxy-substituted phosphorothioate oligonucleotides have recently been reported to be more stable in mice than their parent compounds and to display enhanced oral bioavailability (Zhang et al. 1995; Agrawal et al. 1995). The analogs displayed tissue distribution similar to that of the parent phosphorothioate.

Similarly, we have compared the pharmacokinetics of 2'-propoxy modified phosphodiester and phosphorothioate deoxynucleotides (S.T. Crooke et al. 1996). As expected, the 2'-propoxy modification increased lipophilicity and nuclease resistance. In fact, in mice the 2'-propoxy phosphorothioate was too stable in liver or kidney to measure an elimination half-life.

Interestingly, the 2'-propoxy phosphodiester was much less stable than the parent phosphorothioate in all organs except in the kidney. There the 2'-propoxy phosphodiester was remarkably stable. The 2'-propoxy phosphodiester did not bind to albumin significantly, while the affinity of the phosphorothioate for albumin was enhanced. The only difference observed between the analogs was a slight increase in renal toxicity associated with the 2'-propoxy phosphodiester analog (Henry et al. 1997).

Incorporation of the 2'-methoxyethyoxy group into oligonucleotides increased the T_m by 1.1°C/modification when hybridized to the complementary RNA. In a similar manner, several other 2'-O-alkoxy modifications have been reported to enhance the affinity (Martin 1995). The increase in affinity for these modifications was attributed to (a) the favorable gauche effect of side chain and (b) additional solvation of the alkoxy substituent in water.

More substantial carbohydrate modifications have also been studied. Hexose-containing oligonucleotides were created and found to have very low affinity for RNA (Pitsch et al. 1995). Also, the 4' oxygen has been replaced with sulfur. Although a single substitution of a 4'-thio modified nucleoside resulted in destabilization of a duplex, incorporation of two 4'-thio modified nucleosides increased the affinity of the duplex (Bellon et al. 1994). Finally, bicyclic sugars have been synthesized with the hope that preorganization into more rigid structures would enhance hybridization. Several of these modifications have been reported to enhance hybridization (Sanghvi and Cook 1994).

5. Backbone Modifications

Substantial progress in creating new backbones for oligonucleotides that replace the phosphate or the sugar-phosphate unit has been made. The objectives of these programs are to improve hybridization by removing the negative charge, enhance stability, and potentially improve pharmacokinetics.

For a review of the backbone modifications reported to date, please see S.T. Crooke (1995b) and Sanghvi and Cook (1994). Suffice it to say that numerous modifications have been made that replace phosphate, retain hybridization, alter charge, and enhance stability. Since these modifications are now being evaluated in vitro and in vivo, a preliminary assessment should be possible shortly.

Replacement of the entire sugar-phosphate unit has also been accomplished and the oligonucleotide analogs produced have displayed very interesting characteristics. Peptide nucleic acid (PNA) oligomers have been shown to bind to single-stranded DNA and RNA with extraordinary affinity and high sequence specificity. They have been shown to be able to invade some double-stranded nucleic acid structures. PNA oligomers can form triple-stranded structures with DNA or RNA.

PNA oligomers were shown to be able to act as antisense and transcriptional inhibitors when microinjected in cells (Hanvey et al. 1992). PNA oligonucleotides appear to be quite stable to nucleases and peptidases as well.

In summary, then, in the past 5 years, enormous advances in the medicinal chemistry of oligonucleotides has been reported. Modifications at nearly every position in oligonucleotides have been attempted and numerous potentially interesting analogs have been identified. Although it is far too early to determine which of the modifications may be most useful for particular purposes, it is clear that a wealth of new chemicals is available for systematic evaluation and that these studies should provide important insights into the structure–activity relationships of oligonucleotide analogs.

III. Conclusions

Although there are many more unanswered than answered questions about antisense, progress has continued to be gratifying. Clearly, as more is learned, we will be in the position to perform progressively more sophisticated studies and to understand more of the factors that determine whether an oligonucleotide actually works via an antisense mechanism. We should also have the opportunity to learn a great deal more about this class of drugs as additional studies are completed in humans.

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CHAPTER 2

Antisense Medicinal Chem

P.D. Cook

A. Introduction

Making drugs out of oligonucleotide[illegible] deavor. Intense efforts in this area [illegible] several new biotechnology compan[illegible] research attempt to use oligonucleotide[illegible] antisense strands, ribozymes, triplex[illegible] coys. In the antisense approach, R[illegible] bind an oligonucleotide ligand in [illegible] Phosphorothioate (PS) oligonucleotide[illegible] antisense agents, have been shown to [illegible] and several PSs have progressed to late[illegible] been learned about the pharmacology [illegible] erties of the first generation PS antisense[illegible] certain limitations as antisense agents[illegible] certain that PS drugs will soon become[illegible] improve, the oligonucleotide drug class [illegible] structural changes to PS are required [illegible] tions, at all possible modification sites [illegible] reported. This chemical data base should [illegible] ing medicinal chemistry efforts to[illegible] number of general reviews, primarily [illegible] medicinal chemistry, have periodically [illegible] (Zon 1988; Stein and Cohen 1988; [illegible] 1990; Cook 1991, 1993; Milligan, [illegible] Sanghvi and Cook 1994; Kiely 1994; [illegible] 1996a; Matteucci 1996; Matteucci and [illegible] 1997).

B. Scope of the Review

Of the published reviews concerned [illegible] otides, two general approaches have [illegible] possible oligonucleotide modifications [illegible]

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Antisense Oligonucleotide-based Therapeutics

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I. INTRODUCTION

Antisense oligonucleotides are short synthetic oligonucleotides, usually between 15 and 25 bases in length designed to hybridize to RNA through Watson–Crick base pairing (Fig. 1). Upon binding to the target RNA, the oligonucleotide prevents expression of the encoded protein product in a sequence-specific manner. As the rules for Watson-Crick base pairing are well characterized (1), antisense oligonucleotides represent, in principal, a simple method for rationally designing drugs. In practice, exploitation of antisense oligonucleotides for therapies has presented a unique set of challenges, some anticipated and others unanticipated. Nevertheless, antisense oligonucleotides are showing promise as therapeutic agents broadly applicable for the treatment of human diseases. Currently, there is 1 approved antisense product in the market and at least 20 agents currently in clinical trials, several of which are in advanced stages of development (Table 1). In this chapter, we summarize the properties of antisense oligonucleotides in terms of their application as therapeutic agents. As expected, there is significantly more information regarding first-generation phosphorothioate oligodeoxynucleotides; this serves as a good benchmark for comparison with some of the newer modified oligonucleotides. One antisense mechanism that we do not discuss in this chapter are ribozymes, as they are covered elsewhere in this volume.

II. ANTISENSE MECHANISM OF ACTION

Antisense oligonucleotides are small synthetic oligonucleotides that are designed to bind to mRNA through Watson–Crick hybridization. Upon binding to the RNA, the oligonucleotide may inhibit expression of the encoded gene product through either inducing cleavage of the RNA by RNases such as RNase H or by occupancy of critical regulatory sites on the RNA (Fig. 2). Several studies have documented that phosphorothioate oligodeoxynucleotides promote cleavage of the targeted RNA by a mechanism consistent with RNase H cleavage (2–6). RNase H is a ubiquitously expressed enzyme that cleaves the RNA strand of an RNA–DNA heteroduplex (6,7). If the antisense oligonucleotide use DNA chemistry, it will direct RNase H to specifically cleave the target RNA upon binding.

Another RNase-dependent antisense mechanism that has recently received much attention is interference RNA or RNAi (8–13). Introduction of long double-stranded RNA (dsRNA) into eukaryotic cells leads to the sequence-specific degradation of homologous gene transcripts. The long dsRNA molecules are metabolized to small 21 to 23 nucleotide interfering RNAs (siRNAs) by the action of an endogenous ribonuclease, Dicer (14–16). The siRNA molecules bind to a protein complex, termed RNA-induced silencing complex (RISC), which contains a helicase activity that unwinds the 2 strands of RNA molecules, allowing the antisense strand to bind to the targeted RNA molecule (12,17). The RISC is also believed to contain an endonuclease activity, which hydrolyzes the target RNA at the site where the antisense strand is bound. It is unknown whether the antisense RNA molecule is also hydrolyzed or recycles and binds to another RNA molecule. Therefore, RNA interference is an antisense mechanism of action, as ultimately a single- strand RNA molecule binds to the target RNA molecule by Watson–Crick base pairing rules and recruits a ribonuclease that degrades the target RNA.

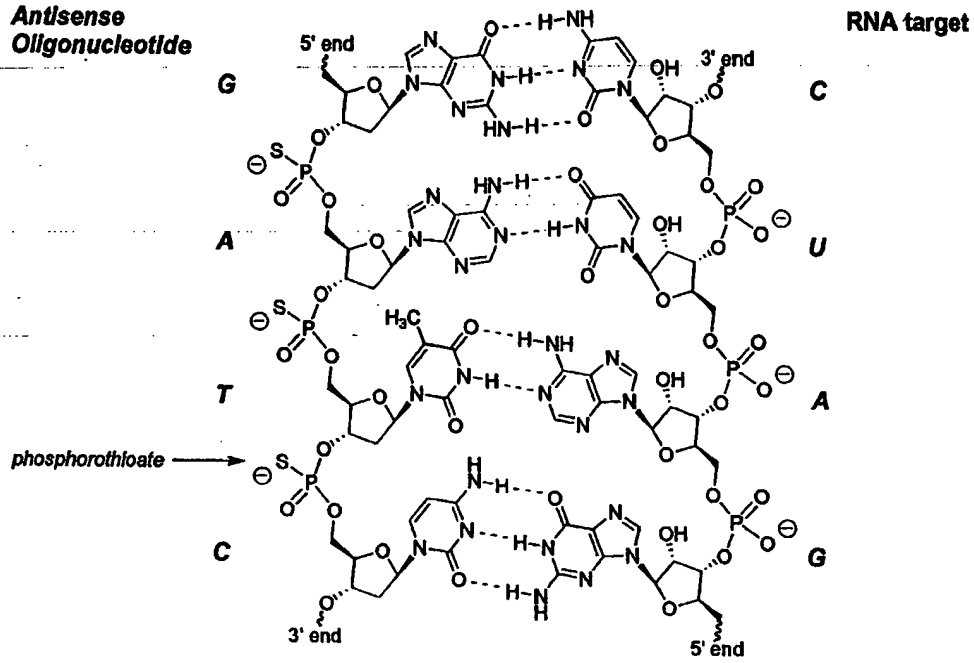

Figure 1 Phosphorothioate antisense oligodeoxynucleotide targeting an RNA receptor. Watson–Crick base pairing rules are indicated: nucleobase adenosine hydrogen bonds to nucleobase uracil, nucleobase cytosine hydrogen bonds to nucleobase guanine.

In mammalian cells, long double-stranded RNA molecules were found to promote a global change in gene expression, obscuring any gene-specific silencing (18,19). This reduction in global gene expression is believed to be mediated in part, through activation of double-stranded RNA-activated protein kinase (PKR), which phosphorylates and inactivates the translation factor eIF2α (20). Recently, it has been shown that transfection of synthetic 21-nucleotide siRNA duplexes into mammalian cells does not elicit the PKR response, allowing effective inhibition of endogenous genes in a sequence-specific manner (21,22). These siRNAs are too short to trigger the nonspecific dsRNA responses, but they still promote degradation of complementary RNA sequences (21–23). We have directly compared the activity of optimized oligonucleotides that work by RNase H mechanism with those that work by an RNAi mechanism in human cells (24). The potency, maximal efficacy, duration of action, selectivity and efficiency for identification of leads was similar for both mechanisms in cell-based assays. The one noted difference between the 2 mechanisms, is that RNase H oligonucleotides are able to cleave pre-mRNA in the nucleus, whereas siRNA oligonucleotides appear to only be able to interact with mature mRNA in the cytoplasm. These results suggest that both mechanisms are equally valid for inhibition of gene expression in mammalian cells.

There are other RNases present in cells that may be exploited in a manner similar to RNase H or the dsRNase associated with RNAi. As an example, Wu et al., reported that single-stranded, phosphorothioate-modified oligoribonucleotides can promote selective loss of ha-ras mRNA in human cells (25). The RNA oligonucleotides could be partially modified with 2′-O-methyl nucleosides and still support enzyme activity. The enzyme activity is consistent with a RNase III type enzyme. RNase III activity is present in both cytosolic and nuclear extracts (25). It is unclear if this enzyme activity is the same RNase III used for siRNA oligonucleotides. Recent work has demonstrated that single-stranded RNA oligonucleotides can interact with the RISC and promote selective degradation of targeted RNA, consistent with RNAi activity, albeit not as efficiently as double-stranded RNA (26,27). Another RNase enzyme that has been exploited for antisense applications is RNase L (28). RNase L is ribonuclease activated by 2′-5′-linked oligoadenylates generated in response to interferon activation. Selectively, linkage of 2′-5′ oligoadenylate to an antisense oligonucleotide has been reported to promote selective cleavage of the targeted mRNA (28–30).

It should be noted that not all oligonucleotide designed to hybridize to a target RNA effectively inhibit target gene expression (2,31–33). This is believed to be due to inaccessibility of some regions of the RNA to the oligonucleotide due to secondary or tertiary structure or to protein interactions with the RNA. At this time, there are no good predictive algorithms for predicting antisense oligonucleotide-binding sites

Table 1 Antisense Oligonucleotides Approved or Currently in Clinical Development

Oligonucleotide	Molecular target	Disease indication	Chemistry	Route of administration	Status	Sponsor
Vitravene (fomivirsen, ISIS 2922)	Human CMV IE-2 gene	CMV retinitis	Phosphorothioate oligodeoxynucleotide	Intravitreal	Marketed	Novartis Ophthalmic/ISIS Pharmaceuticals
LY900003 (Affinitak, ISIS 3521)	Protein kinase C-α	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase III/II	Lilly/ISIS
Oblimersen (Genasense, G3139)	BCL-2	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase III/II	Aventis/Genta
Alicaforsen (ISIS 2302)	ICAM-1	Crohn's disease, ulcerative colitis	Phosphorothioate oligodeoxynucleotide	Intravenous/ enema	Phase III/II	ISIS Pharmaceuticals
ISIS 2503	ha-*ras*	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase II	ISIS Pharmaceuticals
EPI-2010	Adenosine A1 receptor	Asthma	Phosphorothioate oligodeoxynucleotide	Inhaled	Phase II	EpiGenesis
ISIS 14803	HCV RNA	HCV	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase II	ISIS Pharmaceuticals
GTI-2040	Ribonucleotide reductase R1 subunit	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase II	Lorus Therapeutics
GTI-2501	Ribonucleotide reductase R2 subunit	Cancer	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase II	Lorus Therapeutics
LErafAON	c-raf kinase	Cancer-radiosensitizer	Liposome formulation of phosphorothioate oligodeoxynucleotide	Intravenous	Phase I/II	NeoPharm
AP12009	TGF-B2	Malignant glioma	Phosphorothioate oligodeoxynucleotide	Intravenous	Phase I/II	Antisense Pharma
Gem-231	Protein kinase A	Cancer	Phosphorothioate 2′-*O*-methyl/ oligodeoxynucleotide chimera	Intravenous	Phase II	Hybridon
MG98	DNA Methyltransferase	Cancer	Phosphorothioate 2′-*O*-methyl/ oligodeoxynucleotide chimera	Intravenous	Phase II	MethyGene/MGI Pharma/British Biotech
ISIS 104838	TNF-α	Rheumatoid arthritis	Phosphorothioate 2′-*O*-methoxyethyl/ oligodeoxynucleotide chimera	Subcutaneous/ Oral	Phase II	ISIS Pharmaceuticals
OGX-011 (ISIS 112989)	Clusterin	Cancer	Phosphorothioate 2′-*O*-methoxyethyl/ oligodeoxynucleotide chimera	Intravenous/ subcutaneous	Phase I/II	Oncogenix/ISIS Pharmaceuticals
ISIS 113715	PTP-1B	Diabetes	Phosphorothioate 2′-*O*-methoxyethyl/ oligodeoxynucleotide chimera	Subcutaneous	Phase I	ISIS Pharmaceuticals
ATL1102 (ISIS 107248)	CD49D (alpha subunit of VLA4)	Multiple sclerosis	Phosphorothioate 2′-*O*-methoxyethyl/ oligodeoxynucleotide chimera	Subcutaneous	Phase I	Antisense Therapeutics Ltd/ISIS Pharmaceuticals
Resten-NG	c-myc	Restenosis	Morpholino	Catheter delivery-intra-arterial	Phase II	AVI BioPharma
Oncomyc-NG	c-myc	Cancer	Morpholino	Unknown	Phase I/II	AVI BioPharma
AVI-4126	c-myc	Polycystic kidney disease	Morpholino	Intravenous	Phase I	AVI BioPharma
AVI-4557	Cytochrome P450 (CYP3A4)	Inhibit drug metabolism	Morpholino	Intravenous	Phase I	AVI BioPharma

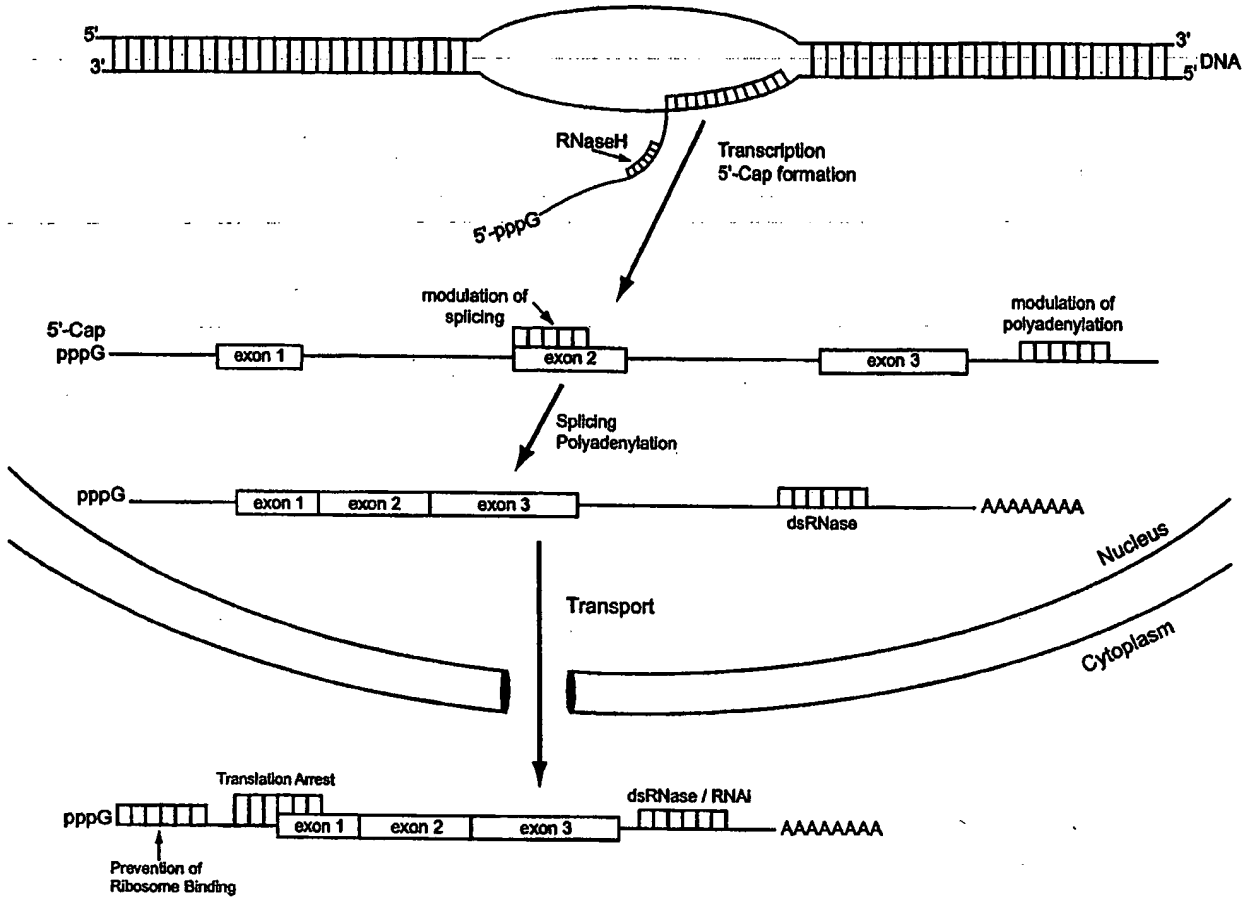

Figure 2 Antisense mechanisms of action. Cartoon depicting 3 different mechanisms by which an antisense oligonucleotide can inhibit expression of a targeted gene product by hybridization to the mRNA, or pre-mRNA which codes for the gene product.

on a target RNA. In our experience, we have found active oligonucleotides that work through an RNase H-dependent mechanism can hybridize to any region on the mRNA or pre-mRNA. Thus, some serendipity is still involved in the process of identifying and optimizing potent and effective antisense inhibitors.

Early on it was believed that occupancy of the RNA (the receptor for the antisense oligonucleotide) by the oligonucleotide would be sufficient to block translation of the RNA (i.e., translation arrest) (34). Subsequent studies have documented that oligonucleotides are not efficient at blocking translation of mRNA if they bind 3′ to the AUG translation initiation codon. Furthermore, we have found that only certain sites in the 5′-untranslated region of a mRNA are effective target sites for an antisense oligonucleotide. In particular, the 5′-terminus of a transcript appears to be a good target site for oligonucleotides for some molecular targets in that occupancy of this region prevents assembly of the ribosome on the RNA (35). It should be noted that occupancy of the receptor (RNA) and steric blocking of factor binding by high-affinity oligonucleotides can be an efficient mechanism for blocking gene expression. For the example cited above, the steric blocking oligonucleotide was approximately 10-fold more potent than an oligonucleotide that supports RNase H activity. These results suggest that catalytic turnover of the target RNA is not the rate-limiting step for antisense oligonucleotides.

Another process that noncatalytic oligonucleotides can use to alter gene expression is through regulating RNA processing. Most mammalian RNAs undergo multiple post- or cotranscriptional processing steps, including addition of a 5′-cap structure, splicing, and polyadenylation. Because single-stranded antisense oligonucleotides localize to the cell nucleus (36–39), they have the potential of regulating these processes.

Several studies have been published documenting that antisense oligonucleotides can be used to regulate RNA splicing in both cell-based assays and in rodent tissues (40–47). Oligonucleotides can be used to modulate alternative splicing by promoting use of cryptic splice sites as was exemplified for β thalassemia (40,41), or by enhancing use of an alternative splice site. Oligonucleotide binding to the pre-mRNA can also be exploited to mask polyadenylation signals on the pre-mRNA, forcing the cell to use alternative poly A sites (48). Finally, oligonucleotides, in principle, can regulate RNA function by sterically preventing factors from binding or changing the structure of the RNA such that it is no longer recognized by the factor. Thus, there are multiple mechanisms by which oligonucleotides can be use to inhibit or modulate expression of a target gene product. No single mechanism is far superior to other mechanisms, thus one should tailor the mechanism for the specific biological application.

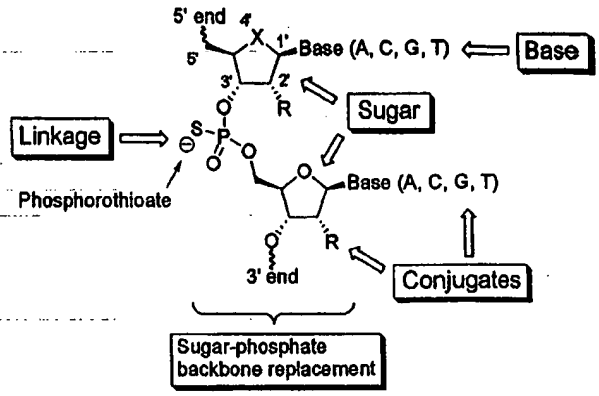

Figure 3 Positions that have been chemically modified for antisense oligonucleotides.

III. ANTISENSE OLIGONUCLEOTIDE CHEMISTRY

The most advanced oligonucleotide chemistry used for antisense drugs is phosphorothioate oligodeoxynucleotides. These differ from natural DNA in that 1 of the nonbridging oxygen atoms in phosphodiester linkage is substituted with sulfur (Fig. 1). Phosphorothioate oligodeoxynucleotides are commercially available, easily synthesized, support RNase H activity, exhibit acceptable pharmacokinetics for systemic and local delivery, and have not exhibited major toxicities that would prevent their use in humans. There have been significant resources employed to identify chemical modifications that further improve upon the properties of phosphorothioate oligodeoxynucleotides. The primary objectives of the effort are similar to medicinal chemistry efforts for other types of pharmacological agents (i.e., to increase potency, improve pharmacokinetics, and decrease toxicity).

A dimer of an oligonucleotide depicting subunits that may be modified to enhance oligonucleotide drug properties is depicted in Fig. 3. In naturally occurring nucleic acids, these subunits are composed of heterocycles, carbohydrate sugars, and phosphodiester-based linkages between the sugars. The combination of the carbohydrate sugar (ribose in RNA, 2′-deoxyribose in DNA) and the linkage provides the backbone of the oligonucleotide polymer. Many modifications have been made on the individual base, sugar, and linkage subunits, and the sugar-phosphate backbone has been completely replaced with an appropriate substitute. In Addition, many diverse moieties have been conjugated to various positions in the subunits, mainly in an attempt to alter the biophysical properties of the polymer. Finally, prodrug modifications may be employed to enhance drug properties. Most of the positions available in a nucleoside dimer (approximately 25 positions for each dimer that do not directly interfere with Watson–Crick base pair–hydrogen bonding) have been modified and studied for their effects on the properties of the resulting oligonucleotides.

The nucleobases or heterocycles of nucleic acids provide the recognition points for the Watson–Crick base pairing rules and any oligonucleotide modification must maintain these specific hydrogen-bonding interactions. Thus, the scope of heterocyclic modifications is somewhat limited. The relevant heterocyclic modifications can be grouped into 2 structural classes: (1) those that enhance base stacking, and (2) those that provide additional hydrogen bonding. The primary objective of heterocyclic modifications being to enhance hybridization, resulting in increased affinity (Fig. 4). Modifications that enhance base stacking by expanding the π-electron cloud are represented by lipophilic modifications in the 5-position of pyrimidines, such as propynes, hexynes, azoles, and a simple methyl group (49–52) and the 7 position of 7-deaza-purines position, including iodo, propynyl, and cyano groups (53–55). Investigators have continued to build out of the 5-position of cytosine by going from the propynes to 5-membered heterocycles to tricyclic fused systems emanating from the 4 and 5-positions of (Fig. 4) (56–59). A second type of heterocycle modification is represented by the 2-aminoadenine (Fig. 4), where the additional amino group provides another hydrogen bond in the A-T base pair, analogous to the 3 hydrogen bonds in a G-C base pair. Heterocycle modifications providing a combination of effects are represented by 2-amino-7-deaza-7-modified A (55) and the G-clamp, a tricyclic cytosine analog having hydrogen-bonding capabilities in the major groove of heteroduplexes (58) (Fig. 4). Furthermore, N2-modified 2-amino purine oligonucleotides have exhibited interesting binding properties (60,61). All these modification are positioned to lie in the major or minor groove of the heteroduplex, do not affect sugar conformation of the heteroduplex, and provide little nuclease resistances, but will generally support an RNase H cleavage mechanism.

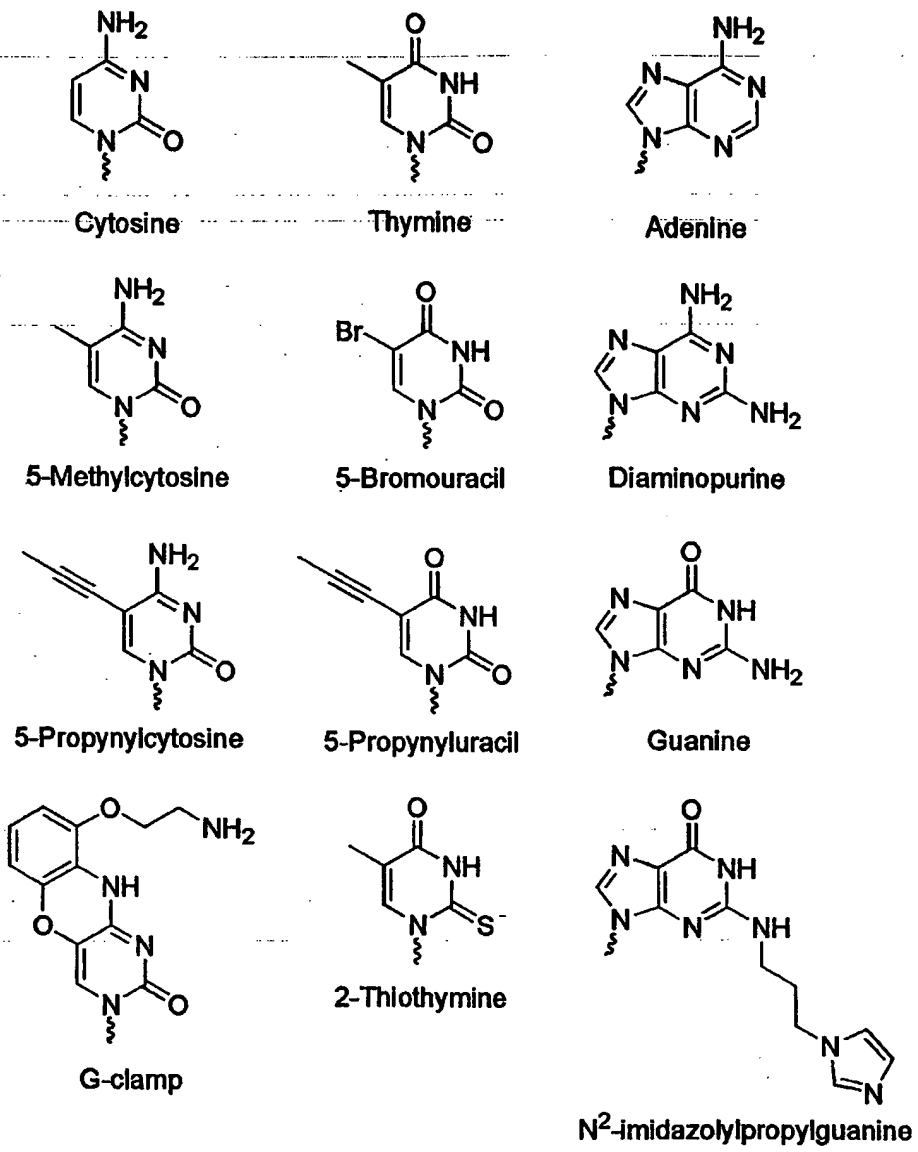

Figure 4 Examples of different heterocycle modifications that support antisense activity.

Modifications in the ribofuranosyl moiety have provided the most value in the quest to enhance oligonucleotide drug properties (Fig. 5). In particular, certain 2′-*O*- modifications have greatly increased binding affinity and nuclease resistance, altered pharmacokinetics, and are potentially less toxic (62). Preorganization of the sugar into a 3′-*endo* pucker conformation is responsible for the increased binding affinity (63–65). The 2′-*O*-methoxyethoxy (MOE) and 2′-*O*-methyl modifications (Fig. 5) are the most advanced of the 2′-modified series, and have entered clinical trials. The cationic 2′-*O*-aminopropyl (66) and 2′-*O*-(dimethylaminooxyethyl) (67,68) have shown favorable binding affinity, with dramatically improved nuclease resistance. In an attempt to extend on the increased nuclease resistance of these cationic modifications to the high affinity seen with MOE, a dimethylaminoethyl version (DMAEOE) was prepared. This modification displays hybridization properties equal to or superior to those of MOE, and nuclease resistance equal to that of the 2′-*O*-aminopropyl modification. The modification showing the largest known improvement in binding affinity is a bicyclic

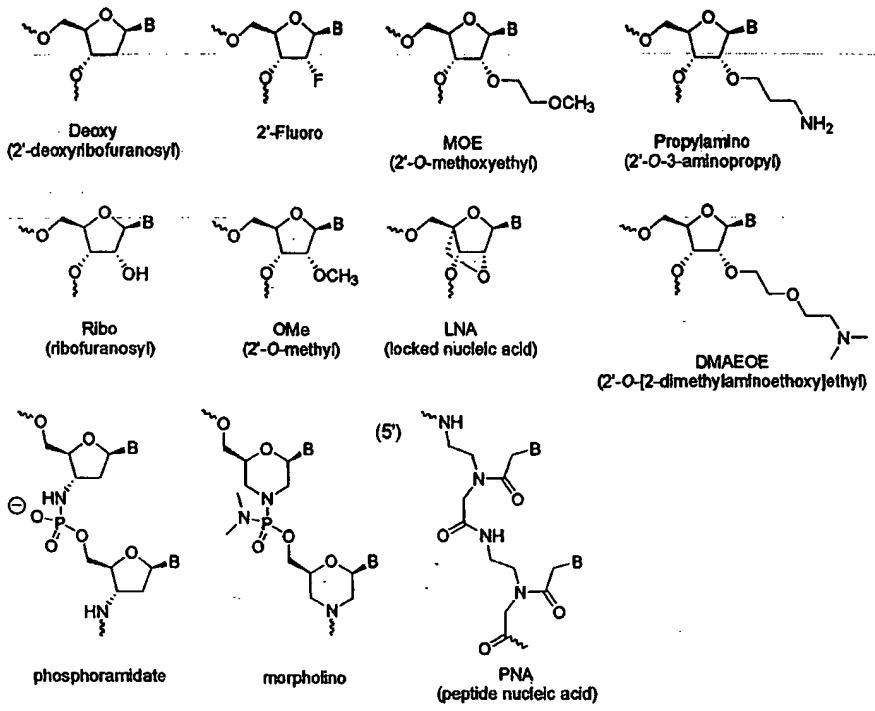

Figure 5 Examples of different sugar and backbone modifications that support antisense activity.

system having the 4′-carbon tethered to the 2′-hydroxyl group. As this modification "locks" the conformation of the ribose sugar into an RNA-like (3′-*endo*) conformation, it is referred to as locked nucleic acid (LNA) (69,70). LNA shows dramatically improved hybridization properties with regard to a reference DNA:RNA duplex, and has extremely high nuclease resistance. Although extremely promising from early biophysical and in vitro data, whether these properties will translate into improved efficacy in vivo remains to be seen.

It is now well known that uniformly 2′-*O*-modified oligonucleotides do not support an RNase H mechanism (71). The heteroduplex formed has been shown to present a structural conformation that is recognized by the enzyme, but cleavage is not supported (72–74). Thus, uniformly modified, "RNA–like" oligonucleotides (3′-*endo* sugar conformation) will be unable to effect cleavage of the target mRNA, and must therefore exert their effects via other means. This has led to the development of a chimeric strategy (3,71,75–77), which focuses on the design of high-affinity, nuclease-resistant antisense oligonucleotides that contain a "gap" of contiguous phosphorothioate- modified oligodeoxynucleotides (Fig. 6). On hybridization to target RNA, a heteroduplex is presented that supports an RNase H-mediated cleavage of the RNA strand via interaction with the 2′-deoxy gap region. The stretch of the modified oligonucleotide–RNA heteroduplex, which is recognized by RNase H may be placed anywhere within the modified oligonucleotide. The modifications in the flanking regions of the gap should not only provide nuclease resistance to exo- and endonucleases, but also not compromise binding affinity and base pair specificity. There are several types of structures that have been successfully developed (Fig. 6), with the most advance being "gapmers," having a 7- to 10-base oligodeoxynucleotide gap flanked by 2 regions of 2′-modified nucleosides. These oligomers, in particular, 2′-MOE modified, show reduced toxicity, increased potency, and superior pharmacokinetics relative to the parent unmodified 20-mer phosphorothioate oligodeoxynucleotide (77–81).

Several possible mechanisms exist for uniformly modified, non-RNase H activating oligonucleotides to show efficacy, such as prevention of assembly of the ribosome through binding in the 5′-UTR, "translation arrest," or ribosome stalling by blocking the reading of the mRNA ribosome, and modulation of splicing events by binding to splice junctions. Although all these strategies have been pursued, no uniformly modified oligonucleotides have advanced beyond gapmer oligonucleotides. However, much recent progress has been made with non-RNase H active oligonucleotides, and there remains much potential for these modifications. LNA and MOE have been used in a uniform context in addition to the gapmer strategy, and early studies show promise. Another interesting uniform

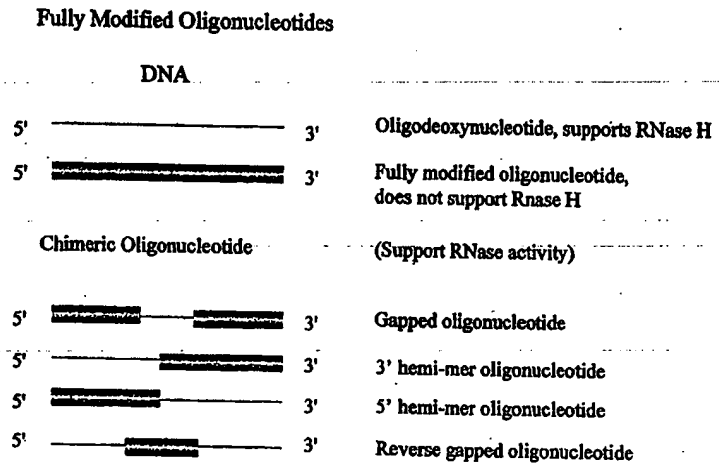

Figure 6 Examples of different oligonucleotide structures.

modification is the phosphoramidiate modification, which substitutes an amino group for the 3′ oxygen atom of the deoxyribose sugar of DNA. This results in a preference for the RNA-like (3′ endo) sugar conformation, and results in increased affinity as is seen with the 2′-*O*-alkyl modifications (82,83).

One of the most intriguing backbone oligonucleotide modifications is peptide nucleic acid (PNA). PNA is unique in that the sugar-phosphate backbone is completely replaced with a peptide-based backbone (Fig. 5) (84). This results in a polymer with a neutral backbone that has high affinity for complimentary nucleic acids. PNA has been extensively investigated as an antisense agent, but these efforts have generally been frustrated by the poor cellular penetration and in vivo pharmacokinetic properties of PNA (85). Recently, a 4-lysine peptide conjugated to a PNA was found to provide robust in vivo activity when targeted to a splice junction (47). These data are highly encouraging because they may provide a path to realizing the promise of PNA as an antisense therapeutic agent.

The most advanced uniform modification is the "morpholino" modification (Fig. 5), which is currently in phase II clinical trials for restenosis, cancer, and polycystic kidney disease. The morpholino modification simultaneously replaces the ribofuranosyl sugar with a morpholine ring, and the negatively charged phosphate ester with a neutral phosphorodiamidate linkage (86,87). Morpholinos are generally used around the translation initiation start codon, and are believed to function via translation arrest. A morpholino oligonucleotide has shown in vivo activity (88), as well as oral bioavailability in rats (89), which would be a major advance if studies proved general and translated to larger mammalian species.

In addition to heterocycle, backbone, and sugar modification discussed above, various pendant groups have been attached to oligonucleotides, such as cholesterol, folic acid, fatty acids, etc., to alter pharmacokinetic properties (90,91). The reader is referred to several recent reviews that discuss the chemistry of oligonucleotides in more detail (92–95). It should be noted that there is no single modification that covers all the desired properties for a modified oligonucleotide. Modifications have been identified that increase hybridization affinity of the oligonucleotide for its target RNA, increase nuclease resistance, decrease toxicity, and alter the pharmacokinetics (Table 2). Furthermore, the ideal oligonucleotide will differ for different applications. Therefore, it is important to be able to mix and match the various modifications to obtain the optimal oligonucleotide for the task at hand.

Table 2 Attributes of Various Modified Oligonucleotides

Attribute	Examples
Increased affinity for RNA	2′-*O*-methyl, 2′-fluoro, MOE, DMAEOE, LNA, 5-MeC, 5-propynyl, phenoxazine G-clamp, PNA, phosphoramidate, others
Increased nuclease resistance	MOE, DMAEOE, LNA, PNA, phosphoramidate, morpholino, others
Alter tissue distribution	MOE, PNA, cholesterol conjugate, phosphoramidate, morpholino, others
Decrease toxicity	2′-*O*-methyl, MOE, 5-MeC, morpholino, others

IV. PHARMACOKINETICS OF OLIGONUCLEOTIDES

A. Cellular Pharmacokinetics

Cellular uptake of phosphorothioate oligonucleotides has been documented to occur in most mammalian cells (96–103). Cellular uptake of oligonucleotide is time and temperature dependent. It is also influenced by cell type, cell culture conditions and media, and the length/sequence of the oligonucleotide itself (96). No obvious correlation between the lineage of cell, whether the cells are transformed or virally infected, and uptake has been identified. Cellular uptake appears to be an active process (i.e., oligonucleotide) will accumulate in greater concentration intracellular than in the medium and is energy dependent. Despite the fact that mammalian cells in culture will readily accumulate oligonucleotides, it has been necessary to further facilitate cytosolic delivery for many, but not all, cells with transfection agents such as cationic lipids, dendrimers, fusogenic peptides, electroporation, etc., (38,46,104–108). In the absence of these facilitators, it has been difficult to demonstrate true antisense effects in cultured cells., although there are some exceptions. However, in vivo, this is not the case. It has become apparent that in vitro cell uptake studies do not predict in vivo cell uptake and pharmacokinetics of oligonucleotides (96,109–113). Our understanding of cellular and subcellular uptake has evolved as superior analytical tools have been developed. These advances include development of immunohistochemical techniques use oligonucleotide-specific antibodies (114), and in situ perfusion of whole organs followed by cell sorting and subcellular separation techniques coupled with capillary gel electrophoresis (110).

Our understanding of cellular and subcellular distribution and pharmacokinetics of oligonucleotides in whole animals is emerging. In our laboratories, we use more specific tools for qualification and even quantification of intact oligonucleotide (110,114–116). Phosphorothioate oligonucleotides rapidly distribute to whole tissue with distribution half-lives range from 30 to 60 min in vivo. Approximately half of the oligonucleotide associated with the liver (as an example) is intracellular in both parenchymal and nonparenchymal cells by 4 h after intravenous administration (110,117). The other half of the organ-associated oligonucleotide appears to be associated with extracellular matrix or interstitium, or loosely bound to the cell membrane. Consistent with this observation, others have shown that phosphorothioates have been localized to connective tissue and can bind to various proteins within these matrices, such as laminin and fibronectin (114,118,119). Some of this matrix- associated oligonucleotide will diffuse to cells over time or be lost to efflux from the organ (114). It is likely that both of these processes are functioning up to 24 h after administration of oligonucleotide. By 24 h after injection of phosphorothioate oligonucleotide, little is seen to be associated with extracellular matrix (114). Thus, it is likely that whole organ pharmacokinetic evaluation after 24 h will parallel cellular clearance kinetics.

Although the in vitro studies fail to predict well which cell types will take up oligonucleotide in vivo, the general trend of variability from cell type to cell type continues to be observed in vivo (114). Based on these results, one would not expect to uniformly inhibit expression of a targeted gene product within a tissue or whole organism, resulting in differential sensitivity of different tissues and cells within tissue to the antisense effect. Subcellular distribution has been shown to be broad, and the extent of cytosolic and nuclear distribution differs between cells (110). In general, the total number of oligonucleotide molecules is greatest in the cytosol. However, because of the much smaller volume of the nucleus, the nucleus may often contain a higher concentration of oligonucleotide than the cytosol.

Nuclease metabolism has been shown to account for the clearance of phosphorothioate oligonucleotide from organs of distribution. Within the cells, the pattern of metabolites appears to be quite similar between cell types and the subcellular compartments (membrane associated, cytosolic, and nuclear). Increasing doses from 5 to 50 mg/kg only moderately decreased metabolism intracellularly, consistent with whole organ data (110).

Several studies have suggested that active uptake processes, including receptor-mediated endocytosis and pinocytosis, are involved in uptake of oligonucleotides in vivo. At very low doses (less than 1 mg/kg), competition of binding for scavenger receptors in vivo altered the whole organ distribution of oligonucleotides in liver but not in kidney (120–122). However, distribution studies conducted in scavenger receptor knockout mice did not show significantly altered intracellular and whole organ distribution of phosphorothioate oligonucleotides (123).

Distribution in the kidney has been more thoroughly studied, and drug has been shown to be present in Bowman's capsule, the proximal convoluted tubule, the brush border membrane, and the renal tubular epithelial cells (114,124). These data suggested that the oligonucleotides are filtered by the glomerulus and then reabsorbed by the proximal convoluted tubule epithelial cells. Moreover, the authors identified a specific protein in the brush border that may mediate uptake. In subsequent studies, the authors have purified the 45-kDa protein, reconstituted it in phospholipid vesicles and demonstrated that it served as a channel allowing nucleic acid to pass through phospholipid bilayers (125). In separate studies, other investigators have shown that, although some oligonucleotide is taken up from the tubular lumen brush border, the distribution to the tubule epithelial cells is predominantly from the capillary serosal side (126). The uptake from capillary circulation may not be receptor mediated. In summary, it is likely that there are multiple processes involved in the uptake of oligonucleotides into cells in vivo. Additional research will be required to further elucidate these mechanisms.

B. Whole Animal Oligonucleotide Pharmacokinetics

1. Phosphorothioate Oligodeoxynucleotides

The plasma pharmacokinetics of phosphorothioate oligodeoxynucleotides are characterized by rapid and dose-dependent

clearance (30–60 min half-life) driven primarily by distribution to tissue and secondarily by metabolism. Urinary and fecal excretion are minor pathways for elimination of phosphorothioate oligonucleotides. Dose-dependent clearance from plasma is predominantly a function of saturable tissue distribution (127,128). Metabolism has been shown to be unchanged in plasma over a large dose range (1–50 mg/kg) and after repeated administration up to 1 month, suggesting that metabolism is neither inhibited or induced by repeat administration (129).

The plasma pharmacokinetics are quite similar between animals and man, and they scale from one species to the next on the basis of body weight, not surface area (129–133). For example, it is possible to show that, when dosed on the basis of body weight, the concentrations of oligonucleotides in plasma administered by a 2-h constant intravenous infusion are similar between humans and monkeys. Thus, it has been possible to predict plasma concentrations in humans from nonclinical pharmacokinetic data.

Phosphorothioate oligonucleotides bind to circulating plasma proteins such as albumin and α-2 macroglobulin (134). The apparent affinity for human serum albumin is low (10–30 μM). Therefore, plasma protein binding provides a repository for these drugs preventing rapid renal excretion. Because serum protein binding is saturable at high concentrations, intact oligonucleotide may be found in urine in increasing amounts as dose rate and/or amount is increased (129, 135,136).

Phosphorothioate oligonucleotides are rapidly and extensively absorbed after intradermal, subcutaneous, intramuscular, or intraperitoneal administration (109,127,137,138). Nonparenteral absorption has been characterized for pulmonary and oral routes of administration. Estimates of bioavailability range from 3% to 20% following intranasal dosing and < 1% by the oral route (139,140). Although it is likely that permeability in the intestine is low, stability of these compounds in the intestine (prior to absorption) may be a rate-limiting factor to oral absorption (141,142). As discussed below, some chemical modifications to the oligonucleotide enhance oral absorption. The metabolic half-life of a 20-mer phosphorothioate oligonucleotide in the rat intestine (in vivo) is less than 1 h (data shown in Section VI).

Phosphorothioates are broadly distributed to all peripheral tissues. Highest concentrations of oligonucleotides are found in the liver, kidney, spleen, lymph nodes, and bone marrow with no measurable distribution to the brain (109,127,129, 135,141). Many other tissues take up smaller amounts of oligonucleotide, resulting in lower tissue concentrations. Phosphorothioate oligonucleotides are primarily cleared from tissues by nuclease metabolism. Rate of clearance differs between tissues with the spleen, lymph nodes, and liver, generally clearing more rapidly than kidney, for example. In general, the clearance rates result in half-lives of elimination ranging from 2 to 5 days in rodents and primates (128,133).

In summary, pharmacokinetic studies of phosphorothioate oligonucleotides demonstrate that they are well absorbed from parenteral sites, distribute broadly to all peripheral tissues, do not cross the blood–brain barrier, and are eliminated primarily by slow metabolism. In short, once-a-day or every-other-day systemic dosing should be feasible. In general, the pharmacokinetic properties of this class of compounds appear to be largely driven by chemistry rather than sequence. Additional studies are required to determine whether there are subtle sequence-specific effects on the pharmacokinetic profile of this class of drugs.

2. Second-generation Oligonucleotides

The plasma pharmacokinetics of 2′-*O*-methyl-, 2′-*O*-propyl-, or 2′-*O*-methoxyethyl-modified oligonucleotides do not differ significantly from their oligodeoxynucleotide congeners (79,80,143,144). Because metabolism plays only a minor role in the plasma distribution kinetics, this modification is expected to do little to alter the distribution and excretion kinetics. Early studies in our laboratory indicate that the binding affinity to serum albumin may be somewhat lessened by 2′-ribose sugar modifications, but the overall capacity of the plasma proteins to bind these oligonucleotides is not significantly changed (Table 3). Therefore, urinary excretion remains a minor route of elimination, and these compounds are broadly distributed to peripheral tissues.

Several of the 2′-ribose sugar modification produces enough of an increase in nuclease resistance that it is possible

Table 3 Serum Albumin Affinity, Whole Plasma Fraction Bound to Proteins (F_b), and Fraction of Dose Excreted in Urine ($f_{excreted}$, 0–24 h) Following Intravenous Administration at 3 mg/kg—Comparison of First- and Second-Generation Chemistries

Compound no.	Chemistry	Kd (μM)	$F_{b\,(\%)}$	$f_{excreted}$
ISIS 2302	PS ODN[a]	17.7	99.2	0.003
ISIS 11159	PS 2′-MOE[b]	29.3	95.5	0.032
ISIS 16952	PO 2′-MOE[c]	>500	79.6	0.45

[a]PS ODN, phosphorothioate oligodeoxynucleotide.
[b]PS 2′-MOE, 2′-O-methoxyethyl ribose modified phosphorothioate (all nucleotides were modified).
[c]PO 2′-MOE, 2′-O-methoxyethyl ribose modified phosphodiester (all nucleotides were modified).

to produce relatively stable oligonucleotides with phosphodiester linkages (Table 2). Thus, this modification allows for elimination or reduction in the number of sulfurs contained in the internucleotide bridge, but these compounds are less stable than their 2′-modified phosphorothioate congeners (145). In addition, as sulfur is removed, plasma protein binding is greatly decreased and rapid removal from plasma by filtration in the kidney increases significantly. This pharmacokinetic characteristic may limit the use of phosphodiester second-generation modified oligonucleotides intended for treatment of systemic disease (79). Alternatively, this pharmacokinetic profile may be ideal for locally administered oligonucleotides because it limits the accumulation of systemically absorbed drug.

Absorption for parenterally administered modified oligonucleotides is consistently rapid and nearly complete. Some of the second-generation modified oligonucleotides have exhibited improved intestinal permeability (141) as well as significantly improved stability in the intestine (142). It is likely this combination of improved biochemical characteristics have led to the observation of improved oral bioavailability (141) for this class of oligonucleotide compounds.

The distribution pattern of the 2′-ribose-modified phosphorothioate oligonucleotides are similar to first-generation phosphorothioates and similarly not altered by changes in sequence. Kidney, liver, spleen, bone marrow, and lymph nodes are the major sites of distribution. The most exciting difference in pharmacokinetics is, not surprisingly, manifested in prolonged terminal elimination half-lives from tissues of distribution. The elimination half-lives appear to be increased nearly 5 to 10-fold, suggesting that once-weekly systemic dosing may be feasible (Table 4).

In summary, pharmacokinetic studies of 2′-modified ribose phosphorothioate oligonucleotides demonstrate that they are well absorbed from parenteral sites, may have improved oral absorption attributes, and distribute broadly to all peripheral tissues. Although stability has been greatly enhanced, nuclease metabolism is likely the primary mechanism for ultimate elimination of these modified oligonucleotides. In short, once-a-week systemic dosing should be feasible and oral administration may be possible in the near future. Additional studies are required to determine whether there are substantial sequence-specific effects on the pharmacokinetic profile of this class of drugs.

Table 4 Summary of Observed Organ Clearance Half-lives (in days) Comparing Second- and First-Generation Chemistries

Organ	2′-Modified phosphorothioate oligonucleotide	Phosphorothioate oligodeoxynucleotide
Kidney cortex	21.7	5.0
Kidney medulla	10.4	3.1
Liver	7.7	2.8
Spleen	8.1	3.3
Lymph nodes	16.5	0.9
Bone marrow	11.5	1.3

V. TOXICOLOGY OF OLIGONUCLEOTIDES

Phosphorothioate oligodeoxynucleotides have been examined extensively in a full range of acute, chronic, and reproductive studies in rodents, lagomorphs, and primates. At high doses, there is a distinctive pattern of toxicity that is common to all phosphorothioate oligodeoxynucleotides (146–149). The remarkable similarity in toxicity with different phosphorothioate oligodeoxynucleotides suggests that, for this class of antisense compounds, toxicity is independent of sequence and is the result of nonantisense-mediated mechanisms. The most probable mechanism of the observed toxicities is the binding of oligodeoxynucleotides to proteins. These nonantisense-mediated pathways are believed to be responsible for most, if not all, of the toxicities associated with the administration of these compounds to laboratory animals. This conclusion is strengthened by studies in which little or no differences in toxicity are observed between pharmacologically active and inactive sequences. Different patterns of toxicity exist between rodents and primates. Understanding the mechanisms behind these differences is crucial to understanding which species best predicts the potential human effects. A comparison of the toxicological profiles of phosphorothioate oligodeoxynucleotides with that of the next generation of phosphorothioate oligonucleotides suggests that some of the chemical class-related toxicities of phosphorothioate oligodeoxynucleotides can be ameliorated by chemical modification.

A number of phosphorothioate oligodeoxynucleotides have been examined in 1 or more of the following battery of genotoxicity assays: Ames test, in vitro chromosomal aberrations, in vitro mammalian mutation (HGPRT locus and mouse lymphoma), in vitro unscheduled DNA synthesis tests, and in vivo mouse micronucleus. In all these assays, the results were negative and there was no evidence of mutagenicity or clastogenicity of these compounds (150).

A. Acute Toxicities

In rodents, the acute toxicity of phosphorothioate oligodeoxynucleotides has been characterized as part of an effort to determine the maximum tolerated dose for in vivo genotoxicity assays. The doses of 3 phosphorothioate oligodeoxynucleotides required to produce 50% lethality (LD50) were estimated to be approximately 750 mg/kg (150).

In primates, the acute dose-limiting toxicities are a transient inhibition of the clotting cascade and the activation of the complement cascade (146,151,152). Both of these toxicities are believed to be related to the polyanionic nature of the molecules and the binding of these compounds to specific protein factors in plasma.

Prolongation of clotting times following administration of different phosphorothioate oligodeoxynucleotides is charac-

terized by a concentration-dependent prolongation of activated partial thromboplastin times (aPTT) (149,153–155). The prolongation of aPTT is highly transient and directly proportional to plasma concentrations of oligodeoxynucleotide and therefore parallels the plasma drug concentration curves with various dose regimens. As drug is cleared from plasma, the inhibition diminishes such that there is complete reversal within hours of dosing. With repeated administration, there is no evidence of residual inhibition. Prolongation of aPTT has been observed in all species examined to date, including human, monkey, and rat. The mechanism of prolongation of aPTT by phosphorothioate oligodeoxynucleotides is believed to be a result of the interaction of the oligonucleotides with proteins. It is well known that polyanions are inhibitors of clotting, and phosphorothioate oligodeoxynucleotides may act through similar mechanisms. If these oligonucleotides inhibit the clotting cascade as a result of their polyanionic properties, then binding and inhibition of thrombin would be a likely mechanism of action. However, the greater sensitivity of the intrinsic pathway to inhibition by phosphorothioate oligodeoxynucleotides suggests that there are other clotting factors specific to this pathway that may also be inhibited. Recent data suggest that there is a specific allosteric inhibition of the tenase complex as well as binding to thrombin (152,156).

In clinical trials with ISIS 2302, normal volunteers and patients were dosed with 2 mg/kg infused over 2 h. This regimen produced total oligonucleotide concentrations of 10 to 15 μg/mL and a concomitant increase in aPTT of approximately 50% (130), which correlates well with in vitro human and animal data. The transient and reversible nature of aPTT prolongation, combined with the relatively small magnitude of the change, makes these effects clinically insignificant for the current treatment doses and regimens.

Activation of the complement cascade by phosphorothioate oligodeoxynucleotides has the potential to produce the most profound acute toxicological effects. In primates, treatment with high doses over short infusion times resulted in marked hematological effects and marked hemodynamic changes that are believed to be secondary to complement activation. Hematological changes are characterized by transient reduction in neutrophil counts, presumably due to margination, followed by neutrophilia with abundant immature, nonsegmented neutrophils (147,151). In a small fraction of monkeys, complement activation was accompanied by marked reductions in heart rate, blood pressure, and subsequently cardiac output. In some animals, these hemodynamic changes were lethal (146,151,157).

There is an association between cardiovascular collapse and complement activation. That is, all monkeys demonstrating some degree of cardiovascular collapse or hemodynamic changes had markedly elevated levels of complement split products. However, the converse is not true, in that only a fraction of the animals with activated complement had cardiovascular functional changes (150). Thus, this observation suggests that there may be sensitive subpopulations or predisposing factors within individual animals that make them susceptible to the physiological sequelae of complement activation. Because of these observed hemodynamic changes, primate studies to monitor for these effects have become part of the normal evaluation of these compounds (158,159). Although complement activation at high doses is consistent and predictable between animals, there is currently little appreciation for the variability in the severity of the associated hemodynamic changes. Although the split product Bb can be used to monitor complement activation, it is C5a (complement split product) that is the most biologically active split product. Preliminary data obtained relating response to complement split product levels indicate that C5a levels are elevated more significantly in some of the more affected animals (150).

The goal of toxicity studies is to characterize the toxicity of compounds and to establish a framework upon which clinical safety studies can be designed. In this regard, it is useful to examine the relationship between plasma concentrations of oligonucleotides and the activation of complement. When Bb concentrations were plotted against the concurrent plasma concentrations of oligodeoxynucleotides in primates, it was apparent that complement was only activated at concentrations of phosphorothioate oligodeoxynucleotides that exceed a threshold value of 40 to 50 μg/mL (151). Bb levels remained unchanged from control values at plasma concentrations below the threshold. Remarkably, this threshold concentration is similar for three 20-mer phosphorothioate oligodeoxynucleotides and for an 8-mer phosphorothioate oligodeoxynucleotide that forms a tetrad complex (160,161). Recent data demonstrate that human serum may be less sensitive to activation than monkey serum, suggesting a species difference in sensitivity. Regardless of small differences, it is clear that clinical dose regimens should be designed to avoid plasma oligodeoxynucleotide concentrations that exceed 40 to 50 μg/mL. To this end, the similarities in plasma pharmacokinetics between monkeys and humans have allowed the design of dose regimens that achieve desired plasma concentration profiles.

The most direct approach for staying below the plasma thresholds for complement activation is to reduce the dose rate by substituting prolonged infusions for bolus injections. In clinical trials with phosphorothioate oligodeoxynucleotides, the drugs are administered either as 2-h infusions or as constant 24-h infusions. At a rate of infusion of 2 mg/kg over 2 h, the Cmax was 8 to 15 μg/mL, still well below the threshold for complement activation (130). Phosphorothioate oligodeoxynucleotides have been administered by intravenous infusion to more than 3000 patients and volunteers without any significant indication of activation of the alternative complement cascade.

1. Modified Oligonucleotides

Chemical modifications to phosphorothioate oligodeoxynucleotides may reduce the potential to activate complement. In one study, cynomolgus monkeys were administered an intravenous infusion over a 10-min period with a 5, 20, or 50 mg/kg dose of a 17-mer phosphodiester oligodeoxynucleotide, Ar177, that had phosphorothioate caps on the 3′ and 5′ termini (154,162). This oligonucleotide is known to have a complex secondary structure. In this experiment, although there was

a dose-related increase in plasma concentrations of Bb, the magnitude of the increases were small in comparison to the known activity of full-phosphorothioate oligodeoxynucleotides (162). Whether this diminished potential to activate the complement cascade is related to the reduction of phosphorothioate linkages or whether it is due to the complex secondary structure of this particular oligodeoxynucleotide was not established by these experiments. Some insight into this question was obtained in a second series of experiments performed with oligonucleotides that contained 2′-*O*-methoxyethyl modifications of the ribose sugar in 12 of the 20 nucleotides (149,150). Cynomolgus monkeys were treated by 10-min intravenous infusion with single doses of 1, 5, or 20 mg/kg of this 20-mer oligonucleotide that was either fully modified phosphorothioate linkages (ISIS 13650) or had phosphodiester wings and a central region of phosphorothioate linkages (9 linkages, ISIS 12854). The termini of both compounds contained six 2′-modified nucleotides. A third unmodified phosphorothioate oligodeoxynucleotide, ISIS 1082, was included as a positive control. The unmodified compound produced marked increases in Bb and severe cardiovascular effect at the dose of 5 mg/kg (30- to 60-fold over baseline). At 5 mg/kg, the aPTT values were 41 and 33 sec for the fully phosphorothioate and partially phosphorothioate 2′-modified oligonucleotides, respectively. In contrast, the unmodified phosphorothioate oligodeoxynucleotide produced an aPTT of 72 sec at the same dose. These data suggest that reduction in the number of phosphorothioate linkages reduced the inhibitory effects on aPTT and the activation of the complement cascade. However, the more important difference was that both 2′-*O*-methoxyethyl compounds were markedly less potent in activating complement than an unmodified oligodeoxynucleotide (D.K. Monteith, P.L. Nicklin, and A.A. Levin, unpublished observations, 1997). Although the safety profile of phosphorothioate oligodeoxynucleotides has proven satisfactory, the acute safety profile of the next generation of oligonucleotides may be improved by modification of the 2′-position of the ribose sugar with an alkoxy such as 2′-*O*-methyl or 2′-*O*-methoxyethyl and by reductions in phosphorothioate linkages.

B. Toxicological Effects Associated with Chronic Exposure

One of the characteristic toxicities observed with repeated exposure of rodents to phosphorothioate oligodeoxynucleotides is a profile of effects that can be described as immune stimulation. The profile is characterized by splenomegaly, lymphoid hyperplasia, and diffuse multiorgan mixed mononuclear cell infiltrates (149). The severity of these changes is dose dependent and most notable at doses equal to or exceeding 10 mg/kg. The mixed mononuclear cell infiltrates consisted of monocytes, lymphocytes, and fibroblasts and were particularly notable in liver, kidney, heart, lung, thymus, pancreas, and periadrenal tissues (148,163–165).

Although immune stimulation in rodents is believed to be a class effect of phosphorothioate oligodeoxynucleotides and not dependent on hybridization, sequence is an important factor in determining immunostimulatory potential (166–169). Immunostimulatory motifs have been described in the literature and involve palindromic sequences and CpG (cytosine-guanosine) motifs (169).

Among the most remarkable features of oligodeoxynucleotide-induced immune stimulation are the species differences. Rodents are highly susceptible to this generalized immune stimulation, whereas primates appear to be relatively insensitive to the effect at equivalent doses. Even 6 months of treatment of cynomolgus monkeys with 10 mg/kg of a 20-mer oligodeoxynucleotide, ISIS 2302, given every other day produced only a relatively mild increase in B cell numbers in spleen and lymph nodes of the primates with no change in organ weights. The mixed mononuclear cellular infiltrates in liver and other organs that are so characteristic of the response in rodents are absent even after long-term exposure in monkeys (149). It is known that rodents are more susceptible to the stimulatory effects of lipopolysaccharides, and much of the immune stimulation produced by oligodeoxynucleotides shares characteristics with lipopolysaccharide stimulation. Assuming results obtained in monkeys can be used to predict stimulation in humans, then the immunostimulatory effects may not be a prominent adverse effect in humans.

It is evident that there are both species and sequence differences involved in immune stimulation and that specific sequences should, if possible, be excluded from oligodeoxynucleotides. In long-term toxicity studies in rodents, the constant cell proliferation associated with immune stimulation may have promoter-like effects and may thus complicate the interpretation of rodent carcinogenicity studies. At this time, there are no reports of toxicity studies longer than 6 months, and the long-term sequelae of immune stimulation in rodents are at present merely speculation. More important, immune stimulation following systemic administration of phosphorothioate oligodeoxynucleotides does not appear to be clinically relevant.

Morphologic changes in the bone marrow of mice were observed after 2 weeks of treatment (3 doses/week) with 100 to 150 mg/kg phosphorothioate oligodeoxynucleotide There was reduction in number of megakaryocytes that was accompanied by a reduction of approximately 50% in circulating platelet counts (164). Reductions in platelets have been observed in rats treated with 21.7 mg/kg ISIS 2105 given every other day (148), but were not observed in primates administered 10 mg/kg. Similarly, a reduction in platelets was observed in mice, but not in monkeys treated for 4 weeks with ISIS 2302 at doses of 100 and 50 mg/kg every other day, respectively. Similar observations were made for ISIS 5132 with reductions in platelets at 20 and 100 mg/kg in mice and no observed effect in monkeys up to 10 mg/kg (163). These data suggest that the mouse may be more sensitive to these subchronic effects on platelets than nonhuman primates. However, in acute studies in primates, transient reductions in platelets are occasionally observed. These transient reductions in platelets occur acutely during 2-h infusions at doses of 10 mg/kg, reverse after completion of the infusion, and have not been associated with any measurable change in platelet number 24

to 48 h after subchronic or chronic treatment regimens (150). Thrombocytopenia has been reported in AIDS patients treated with GEM 91, a 27-mer phosphorothioate oligodeoxynucleotide (170).

Tissue distribution studies have shown that the liver and kidney are major sites of deposition of phosphorothioate oligodeoxynucleotide. In toxicity studies with phosphorothioate oligodeoxynucleotides, a variety of hepatic changes have been observed. The immune-mediated cellular infiltrates in rodent livers were discussed above. With high-dose administration of oligodeoxynucleotides in all species examined, there was a hypertrophic change in Kupffer cells accompanied by inclusions of basophilic material that was observed with hematoxylin and eosin staining. These basophilic granules have been identified as inclusions of oligodeoxynucleotide (114). Furthermore, it was demonstrated that the presence of these inclusions was related to dose.

Hepatocellular changes were not a prominent feature of toxicity in primates. In cynomolgus monkeys, 50 mg ISIS 2302 per kg administered every other day for 4 weeks by intravenous injection produced no morphologic indication of liver toxicity, although there was a slight (1.5-fold) increase in AST in this group (171). Following subcutaneous doses of ISIS 3521 and ISIS 5132 of up to 80 mg/kg every other day for 4 doses, there was Kupffer cell hypertrophy and periportal cell vacuolation, but no indication of necrosis and only a very slight increase in ALT (150). After 4 weeks of alternate-day dosing with 10 mg/kg via 2-h intravenous infusion of either ISIS 3521 or ISIS 5132, there were no alterations in AST or ALT, suggesting that at clinically relevant doses of these compounds, there was no evidence for hepatic pathology or tansaminemia. In clinical trials with ISIS 2302, ISIS 3521, and ISIS 5132 at doses of 2 mg/kg administered by 2-h infusion on alternate days for 3 to 4 weeks, there was no indication of hepatic dysfunction, nor was there any evidence of transaminemia.

Like Kupffer cells in the liver, renal proximal tubule epithelial cells take up oligodeoxynucleotide, as demonstrated by autoradiographic studies and immunohistochemistry as discussed previously (114,118,172,173) and by the use of special histologic stains (147). The appearance of basophilic inclusions is dose dependent in proximal tubule cells. Significant renal toxicity can be induced by extremely high doses. Doses of 80 mg/kg in rats and monkeys have induced both histologic and serum chemistry changes in the kidney (174). At clinically relevant doses, however, there was no indication of renal dysfunction. In 4-week or 6-month toxicity studies with phosphorothioate oligodeoxynucleotides, we observed a much more subtle type of morphologic change in the kidney. At a dose of 10 mg/kg on alternate days, here was a decrease in the height of the brush border and enlarged nuclei in some proximal tubule cells. These changes have been characterized as minimal to mild tubular atrophic and regenerative changes. At a dose of 3 mg/kg and below, these changes were only infrequently observed, if at all.

An important aspect of dose-dependent effects is characterization of exposure concentrations and their relationship to morphological changes. To assess exposure, concentrations of oligodeoxynucleotides have been measured in the renal cortex obtained in subchronic and chronic toxicity studies. Renal concentrations increase with increasing doses. The concentration of total oligodeoxynucleotide in the renal cortex associated with minimal to mild (although not clinically relevant) renal tubular atrophy or regenerative changes is approximately 1000 μg/g of tissue. The cortex concentrations of total oligodeoxynucleotide that are associated with moderate degenerative changes after subcutaneous doses of 40 to 80 mg/kg are greater than 2000 μg/g. At a clinically relevant dose of 3 mg/kg every other day, the steady-state concentration of total oligodeoxynucleotide in the kidney is in the range of 400 to 500 μg/g, thus demonstrating a significant margin of safety between the clinical doses and those doses associated with even the most minimal morphologic renal changes. Application of clearance and steady- state pharmacokinetic models suggests that continued administration of oligodeoxynucleotide at this dose should never achieve the renal concentrations associated with dysfunction (129). These models have been confirmed in 6-month chronic toxicology studies, where tissue concentrations measured at the end of 6 months of every-other-day dosing was no different than levels observed after 4 weeks of dosing at a similar or equivalent dose.

C. Chemical Modification of Oligodeoxynucleotides

Chemical modifications of oligodeoxynucleotides have been shown to reduce the potency of immune stimulation. The simplest modification with remarkable activity for reducing the immunostimulatory effects of oligodeoxynucleotides is the replacement of cytosine with 5-methyl cytosine. The methylation of a single cytosine residue in a CpG motif reduced [^{3}H]uridine incorporation and IgM secretion by mouse splenocytes. Methylation of a cytosine not in a CpG motif did not reduce the immunostimulatory potential (175). In our experience with mice, when sequences with 5-methyl cytosine are compared with the same sequence without methylation, the methylated sequence has a lower potency for inducing immune stimulation, as determined by spleen weights and immune cell activation (176,177).

Substitution of methylphosphonate linkages for phosphorothioate linkages on each of the 3′ and 5′ termini have also been reported to reduce the proliferative effects and the secretion of IgG and IgM compared 2 with the full phosphorothioate analog (178). This suggests that that this modification can also be used to ameliorate immune stimulation. The addition of 2′-*O*-methyl substituents also reduced immunostimulatory potential (178). The relative contribution of the uridine substitution and the 2′-methoxy substitution could not be differentiated in this experiment. The effect of 2′-alkoxy modifications on immunostimulatory potential needs further investigation. Finally, the effects of chemical modifications of phosphorothioate oligonucleotides on renal and hepatotoxicity are currently being investigated.

VI. OLIGONUCLEOTIDE FORMULATIONS

A. Physical-chemical Properties

Due to the presence of a mixture of diastereisomers, phosphorothioate oligodeoxynucleotides are amorphous solids possessing the expected physical properties of hygroscopicity, low-bulk density, electrostatic charge pick up, and poorly defined melting point prior to decomposition. Their good chemical stability allows storage in the form of a lyophilized powder, spray-dried powder or a concentrated, sterile solution; more than 3 years of storage is possible at refrigerated temperatures.

Due to their polyanionic nature, phosphorothioate oligodeoxynucleotides are readily soluble in neutral and basic conditions. Drug-product concentrations are limited (in select applications) only by an increase in solution viscosity. The counter ion composition, ionic strength, and pH also influence the apparent solubility. Phosphorothioate oligodeoxynucleotides have an apparent pKa in the vicinity of 2 and will come out of solution in acidic environments (i.e., the stomach). This precipitation is readily reversible with increasing pH or by acid-mediated hydrolysis.

Instability of phosphorothioate oligodeoxynucleotides have been primarily attributed to 2 degradation mechanisms: oxidation and acid-catalyzed hydrolysis. Oxidation of the (P=S) bond in the backbone has been observed at elevated temperatures and under intense ultraviolet light, leading to partial phosphodiesters (still pharmacologically active) and are readily monitored by anion-exchange high-performance liquid chromatography. Under acidic conditions, hydrolysis reactions followed by chain-shortening depurination reactions have been documented by length-sensitive electrophoretic techniques.

B. Parenteral Injections

Given the excellent solution stability and solubility possessed by phosphorothioate oligodeoxynucleotides, it has been relatively straightforward to formulate the first-generation drug products in support of early clinical trials. Simple, buffered solutions have been successfully used in clinical studies by intravenous, intradermal, and subcutaneous injections. Recently, the intravitreal route was approved for the first antisense drug application.

C. Topical Delivery for Diseases of the Skin

The barrier properties of human skin have been an area of multidisciplinary research for a long time. Skin is one of the most difficult biological membrane to penetrate, primarily due to the presence of stratum corneum (SC), which is composed of corneocytes laid in a brick-and-mortar arrangement with layers of lipid. The corneocytes are partially dehydrated, anuclear, metabolically active cells completely filled with bundles of keratin with a thick and insoluble envelope replacing the cell membrane (179). The primary lipids in the SC are ceramides, free sterols, free fatty acids, and triglycerides (180), which form lamellar lipid sheets between the corneocytes. These unique structural features of SC provide an excellent barrier to penetration of most molecules.

Therefore, as the primary barrier to transport of molecules to the skin, physical alteration in SC can result in improved skin penetration. Tape stripping and abrasion by repeated brushing reduced the SC barrier sufficiently to allow penetration of naked plasmid DNA and produced gene expression in skin at a level comparable to that after intradermal injection of naked plasmid DNA (181). Other studies have also shown an increase in oligonucleotide penetration upon physical removal of SC barrier (182–184).

1. Altering the Thermodynamic Properties of the Molecules

Increasing lipid partitioning to improve skin penetration has been evaluated using 2 techniques that alter the thermodynamic properties of oligonucleotide molecules. A complex of phosphorothioate oligonucleotide with hydrophobic cations such as benzalkonium chloride resulted in increased penetration through isolated hairless mouse skin that was explained on the basis of greater partitioning in lipid phase (184). Chemical modification of oligonucleotides to eliminate the negative charges also resulted in a size-dependent increase in the penetration of oligonucleotide into the skin when used with chemical penetration enhancers such as ethanol and dimethyl sulfoxide (183).

2. Electrical Field for Alteration of Skin Permeability

Iontophoresis, which involves application of electric field across the skin to induce electrochemical transport of charged molecules, is studied extensively for transdermal delivery of phosphorothioate oligonucleotides (185,186). The transdermal delivery was shown to be size dependent with steady-state flux values ranging from 2 to 26 pmol/cm^2 in isolated hairless mice skin. The steady-state flux also depended on the sequence, and not just the base composition, of the oligonucleotide. Molecular structure, therefore, is a key contributor to iontophoretically assisted transport of oligonucleotides (187–189). Electroporation a technique using much higher voltage than iontophoresis to cause formation of transient aqueous pathway in skin lipids, provides therapeutic levels (> 1 μM) of oligonucleotides in the viable tissues of the skin (190).

3. Formulations for the Alteration of Skin Permeability

Chemical penetration enhancers have recently been studied for increasing transdermal delivery of oligonucleotides or other polar macromolecules. Chemical-induced transdermal penetration results from a transient reduction in the resistance of the SC barrier properties. The reduction may be attributed to a variety of factors such as opening of intercellular junctions due to hydration (191), solubilization of SC lipids (192,193) or increased lipid bilayer fluidization (194). Types of chemicals known to be penetration enhancers include alkyl esters (195),

phospholipids (196), terpenes (197), nonionic surfactants (198), and laurocapram (Azone) (199). A combination of various surfactants and cosolvents can be used to achieve skin penetration with therapeutically relevant concentration of phosphorothioate oligonucleotides in the viable epidermis and dermis (200). The topical formulations produced significantly higher epidermal and dermal levels of oligonucleotide than those achieved by an intravenous injection at highest tolerated doses. This suggests that the topical route is more efficient in reaching all layers of the skin than systemic administration of phosphorothioate oligonucleotides.

Liposomes have been studied to transport oligonucleotides into the skin. They can increase the fluidity of skin lipid layers (similar to chemical enhancers) to facilitate transdermal permeation and can also carry encapsulated molecules through appendageal pathway (201,202). Mixture of a phosphorothioate oligodeoxynucleotide with a suspension of anionic or neutral lipids resulted in a slight increase in accumulation in epidermis and dermis (R. Mehta, unpublished, 1999). Using a combination of different delivery techniques and formulations, it appears to be feasible to deliver a therapeutically relevant amount of antisense oligonucleotide to the skin. In addition, preliminary results in our laboratory show a dose-dependent pharmacological effect consistent with the antisense mechanism of action of an ICAM-1 antisense oligonucleotide, ISIS 2302 (200). Studies are also underway to assess pharmacology and tissue kinetics of ISIS 2302 in human disease models.

D. Oral Delivery

Of the numerous barriers proposed by Nicklin and others (138) to the oral delivery of oligonucleotides, our experience has confirmed that 2 stand out as critical: instability in the gastrointestinal (GI) tract and low permeability across the intestinal mucosa. Given the formidable nature of these 2 barriers, it is not surprising that oral delivery of oligonucleotides has been considered impossible, or at best, difficult—as is the case with proteins, which has necessitated the latter's nonenteral administration in order to achieve systemic concentrations considered therapeutic. Nevertheless, progress has been made to address and/or understand each barrier with respect to oligonucleotides. (P = S)-oligonucleotides have a distinct advantage over proteins in that the former does not rely on secondary structure for activity. This provides freedom from concern over secondary structure destabilization and allows for (P = S)-oligonucleotide structural modifications to address both presystemic and systemic metabolism.

Natural DNA and RNA are rapidly digested by the ubiquitous nucleases found within the gut. As a consequence, oligonucleotides need to be stabilized in order to achieve a reasonable GI residence time to allow for absorption to occur. Surprisingly, phosphorothioate oligodeoxynucleotides were found to be rapidly degraded by nucleases found in the GI tract; therefore, additional protection from nuclease degradation is required to achieve significant oral bioavailability. Oligonucleotides that are uniformly modified or modified on the 3′-end (gapmers or 3′-hemimers) (Fig. 6) with nuclease-resistant modification have the potential to exhibit increased oral bioavailability. This was demonstrated for both backbone modifications (methylphosphonates) and for sugar-modified (2′-*O*-methyl) oligonucleotides (141,203). We have found that 2′-*O*-methoxyethyl-modified oligonucleotides also exhibit increased oral absorption compared with phosphorothioate oligodeoxynucleotides (80,142).

The physicochemical properties of phosphorothioate oligodeoxynucleotides present a significant barrier to their GI absorption into the systemic circulation or the lymphatics. These factors include their large size and molecular weight (i.e., up to 6.5 kDa for 20-mers), hydrophilic nature (log $D_{o/w}$ approximating −3.5) and multiple ionization pk_as (e.g., G. Hardee, 1999, unpublished titration data, using a Sirius GlpKa instrument on a 20-mer sequence, noted over 17 pk_as for phosphorothioate oligodeoxynucleotide and over 32 pk_as for the 2′-*O*-methoxyethyl hemi-mer form). The use of formulations can improve upon GI permeability. Oligonucleotide drug formulations designed to improve oral bioavailability need to consider the mechanism of oligonucleotide absorption—either paracellular via the epithelial tight junctions, or transcellular via direct passage through the lipid membrane bilayer. By using paracellular and transcellular models appropriate for water-soluble, hydrophilic macromolecules, it was determined that oligonucleotides predominantly traverse GI epithelium via the paracellular route. In this regard, formulation design considerations involve the selection of those penetration enhancers (PEs) that facilitate paracellular transport and meet other formulation criteria, including suitable biopharmaceutics, safety considerations, manufacturability, physical and chemical stability, and practicality of the product configuration (i.e., regarding production costs, dosing regimen, and patient compliance, etc.). Work is in progress, optimizing oligonucleotide chemistry with various permeation enhancers (142,204,205). Preliminary data are encouraging and support continued investment of resources on this endeavor.

E. Liposome Formulations

Liposome formulations of antisense oligonucleotides offer several potential advantages over saline phosphorothioate oligodeoxynucleotides, such as decreased toxicity, altered tissue and cellular distribution, and more convenient dose schedule for the patient. Interesting progress has been reported regarding the passive targeting of oligonucleotides to specific tissues using liposome-encapsulated therapeutics. Accumulation at sites of infection, inflammation, and tumor growth has been attributed to increased circulation times of these materials and the leaky vasculatures associated with these processes (206,207). One caution regarding these observations is worth noting. Because the mononuclear phagocyte system (MPS) is largely responsible for clearing these materials from circulation, misleading data regarding circulation time may be obtained in species with less-evolved systems (i.e., rodents).

Cationic liposomes bind to oligonucleotides due to the electrostatic interaction between positively charged head

groups on lipids and negatively charged phosphates on oligonucleotides. Using the technique of complexation, all the oligonucleotide can be entrapped and purification is not required. The utility of in vivo delivery of oligonucleotide using cationic lipid is limited due to sequestration of material in lung and the RES system (144,208). In addition, interaction of the complex with blood components leads to serum sensitivity and cytotoxicity (209,210).

There are few examples of oligonucleotide delivery by anionic or charge-neutral liposomes. Oligonucleotides encapsulated into cardiolipin-containing anionic liposomes were shown to be taken up 7 to 18-fold more in human T leukemia and ovarian carcinoma cells in vitro. The intracellular release of oligonucleotides was also facilitated and the majority of oligonucleotide was delivered into liposomes (211,212). Methylphosphonate analogs were incorporated into DPPC-containing liposomes and targeted against the Bcr-abl neogene found in chronic myelogenous leukemia (CML). The liposomal- encapsulated oligonucleotides inhibited the growth of CML cells (213). Cellular uptake of oligonucleotides against epidermal growth factor (EGF) encapsulated in DPPC:CHOL liposome containing folate was 9 times higher than nonfolate liposomes and 16 times higher than unencapsulated liposomes (214). There are 2 limitations to intracellular delivery of oligonucleotides by anionic or neutral liposomes: (1) not all cells take up particulate matter, and (2) these liposomes have low encapsulation efficiency.

There is only 1 report of using anionic liposomes in vivo to deliver oligonucleotides. Ponnappa et al. described liposomes consisting DPPC:CHOL:DMPG targeted toward Kupffer cells (215). In this study, greater than 65% of the liver-associated oligonucleotide was found in Kupffer cells.

Conjugation of antibodies to liposomes have been used for targeting of oligonucleotides to specific targets (216–220). Problems with the approach include the inhibition of cellular uptake by the high molecular weight antibody, cost, and poor encapsulation efficiency.

The primary mechanism for cell internalization of neutral liposomes is by endocytosis with the vesicles and their contents delivered to lysozomes (221). pH-sensitive liposomes have been designed to fuse with the endosomes at low endosomal pH and empty their content into cytosol. These pH-sensitive liposomes have been used to deliver antisense oligonucleotides. pH-sensitive liposomes composed of oleic acid: DOPE:Chol-encapsulating antisense oligonucleotide targeted against friend retrovirus inhibited the viral spreading, whereas free oligonucleotide and non pH-sensitive liposomes were ineffective (222,223). pH-sensitive liposomes encapsulating the anti-env oligonucleotide were found to inhibit viral spread at low concentration in infected Dunni cells (224). The major limitation of pH-sensitive liposomes in vivo is their instability in plasma (225,226). This problem was overcome by adding polyethylene glycol-phosphatidylethanolamine (PEG-PE) into the formulation (227). PEG-PE is believed to coat the surface of liposomes, thereby preventing the interaction of liposomes with blood components. This reduced interaction leads to increased stability and plasma half-life of liposomes. The pH-sensitive liposomes composed of CHEMS:DOPE: PEG-PE, when injected intravenously into rats, had similar pharmacokinetics parameters as non pH-sensitive sterically stabilized liposomes. The regular pH-sensitive liposomes without PEG-PE were cleared rapidly from the circulation.

Looking past the question of uptake, a novel approach to releasing endosomal contents into the cytoplasm after uptake has been recently reported (228–230). A 58-kDa protein isolated from Listeria monocytogenes was incorporated into pH-sensitive fluorescent dye. It could be determined that as soon as the endosome began to acidify, the liposome/endosome contents were released into the cytosol. As with the other delivery systems mentioned above, the eventual usefulness of a particular approach will be determined in the near future as we further define the mechanisms and governing restrictions for the inter- and intracellular trafficking of oligonucleotides.

VII. CLINICAL EXPERIENCE WITH ANTISENSE OLIGONUCLEOTIDES

More than 20 different antisense oligonucleotides are currently in clinical trials or approved for use in humans (Table 1). Similar to any other class of drugs, it can be expected that there will be failures in the clinic due to a variety of reasons, such as selection of the wrong molecular target resulting in lack of efficacy, incorrect dosing, marketing consideration, toxicity, etc. It is hoped that because of the generic pharmacokinetics and chemical class-specific toxicity that the failure rates for antisense oligonucleotides will be lower than other classes of agents. However, this remains to be seen.

A. Use of Antisense Oligonucleotides as Antiviral Therapy

The most advanced antisense product is Vitravene® (fomivirsen, ISIS 2922), which is marketed in the United States for the treatment of patients with cytomegalovirus (CMV) retinitis. Fomivirsen was identified from a screen of a series of phosphorothioate oligodeoxynucleotides targeting human cytomegalovirus (HCMV) DNA polymerase gene, or to RNA transcripts of the major immediate-early regions 1 and 2 (IE1 and IE2) (231). Fomivirsen is a 21-mer phosphorothioate oligodeoxynucleotide targeting the coding region of the immediate early 2 gene. Fomivirsen inhibits viral protein expression, as measured by an enzyme-linked immunosorlent assay detecting an HCMV late protein product, in fibroblasts with an EC_{50} value of 0.1 μM. Noncomplementary phosphorothioate oligodeoxynucleotides exhibit an EC_{50} value of 2 μM, 20-fold higher than fomivirsen. In a plaque reduction assay, fomivirsen exhibited an IC_{70} value of 0.1 μM, whereas a control oligonucleotide exhibited an IC_{70} value of 2 μM. These data suggest that HCMV infection of human dermal fibroblast can be inhibited nonspecifically by higher concentrations of phosphorothioate oligodeoxynucleotides: however, fomivirsen is approximately 20-fold more effective than nonspecific oligonucleotides. Fomivirsen reduced IE1 and IE2 proteins in in-

fected cells, as did control oligonucleotides at 10-fold higher concentrations. As the IE1 and IE2 gene products arise from a common pre-mRNA, these results suggest that the oligonucleotide hybridizes to the pre-mRNA. Deletion of sequences from the 5′- and/or 3′-end of the oligonucleotides reduced antiviral activity, whereas introduction of mismatches in the interior of the oligonucleotide did not significantly reduce antiviral activity, although they did reduce hybridization to the target RNA. These data suggest that the antiviral activity of fomivirsen may not be due entirely to an antisense effect. To address this issue in more detail, U373 cells permanently transfected with the IE72 or IE55 polypeptides (derived from the IE1 and IE2 genes, respectively) were treated with fomivirsen (232). Fomivirsen reduced IE55 but not IE72 protein and RNA levels in a sequence-specific manner, suggesting that reduction of IE55 expression occurs by an RNase H-dependent mechanism. As the construct used to express IE72 protein does not contain the fomivirsen-binding site, these data would support that fomivirsen reduces IE55 expression by an antisense mechanism of action. The antiviral activity of fomivirsen was not due to immune stimulation by the CpG motifs in the oligonucleotide (167), as methylation of all the cytosines or only 2 cytosines in the CpG motifs did not reduce antiviral activity. These studies in aggregate suggest that fomivirsen is a potent inhibitor of CMV replication, which is capable of inhibiting viral gene expression by an antisense mechanism of action, but also may inhibit viral replication by a nonantisense mechanism of action at higher concentrations. Whether both mechanism of action are operational in the clinic remains to be elucidated.

Fomivirsen is approved for the local treatment of CMV retinitis in patients with acquired immunodeficiency syndrome, who are intolerant of or have a contraindication to other treatments of CMV retinitis (233,234). The recommended dose is 330 μg every other week for 2 doses and then a maintenance dose administered every 4 weeks given as an intravitreal injection. The most frequently observed adverse event reported for fomivirsen is ocular inflammation (uveitis), including iritis and vitritis (235). Ocular inflammation has been reported to occur in approximately 25% of the patients. Topical corticosteroids have been useful in treating the ocular inflammation. Open-label, controlled clinical studies have been performed, evaluating the safety and efficacy of fomivirsen in newly diagnosed CMV retinitis patients. Based on assessment of fundus photographs, the median time to progression was approximately 80 days for patients treated with fomivirsen, compared with 2 weeks for patients not receiving treatment (234). Although the market for CMV retinitis is relatively small, this drug represents an important validation for the technology.

Gem 132, a second-generation chimeric molecule targeting the HCMV UL36 gene product, is a 20-mer oligonucleotide containing two 2′-*O*-methyl nucleosides on the 5′-end of the molecule and four 2′-*O*-methyl nucleosides on the 3′-end, with the center 14 residues being oligodeoxynucleotides (236). The 2′-*O*-methyl residues also confer increased hybridization affinity and increased nuclease resistance, whereas the center oligodeoxynucleotide residues support RNase H activity. Gem 132 is being evaluated in CMV retinitis patients as both an intravenous infusion and as a direct intravitreal injection. In healthy volunteers, single 2-h infusions of GEM 132 were administered at doses ranging from 0.125 mg/kg to 0.5 mg/kg. Similar to phosphorothioate oligodeoxynucleotides, the plasma pharmacokinetics of GEM 132 were nonlinear with respect to dose. As a single dose up to 0.5 mg/kg, GEM 132 was well tolerated in normal volunteers, with headache being the most frequently reported side effect (237).

Gem 91, a 25-mer phosphorothioate oligodeoxynucleotide designed to hybridize to a conserved region of *gag* human immunodeficiency virus region of (HIV) RNA (238). GEM 91 inhibits viral replication in short-term viral assays in a concentration-dependent manner, whereas a 4- to 5-fold higher concentration of a random mixture of 25-mer phosphorothioate oligodeoxynucleotides (complexity = 4^{25} unique molecules) was required to inhibit viral replication to a similar extent (239). Other studies have demonstrated that acute HIV viral assays are particularly sensitive to the nonantisense effect of phosphorothioate oligodeoxynucleotides (240–243). In chronic HIV assays, GEM91 suppressed viral replication for greater than 30 days, whereas the random mixture of oligodeoxynucleotides only suppressed viral replication for 10 days. GEM 91 was found to be effective against several viral isolates in primary lymphocytes and macrophages, and exhibited selectivity in comparison to the random mixture. In that a random mixture of 4^{25} sequences was used as a control, it is difficult to conclude that GEM 91 inhibits viral replication in a sequence-specific manner. Based on these data, it is likely that at least part of the antiviral activity exhibited by GEM 91 is due to a nonantisense effect.

Phase I/II clinical studies were initiated for GEM 91 in the United States and France (236). The study performed in the United States was a randomized double-blind, placebo-controlled, dose-escalating study in which GEM 91 was administered as a continuous intravenous infusion for 2 weeks, whereas in the French study, GEM 91 was given as a 2-h intravenous infusion every other day for 28 days. Dose levels up to 4.4 mg/kg/day were achieved in the continuous infusion trials, whereas dose levels of 3.0 mg/kg/day were reported for the intermittent infusion trial. Plasma half-lives for GEM 91 were biphasic with mean half-lives of 0.18 h and 26.7 h (236,244). Hybridon recently announced the termination of clinical studies with GEM 91 based on lack of efficacy as measured by viral burden and the development of thrombocytopenia in some of the patients.

ISIS 14803 is a phosphorothioate oligodeoxynucleotide targeting the translation initiation codon of hepatitis C virus (245,246). ISIS 14803 differs from previous phosphorothioate oligodeoxynucleotides in that the cytosines were modified to 5 methyl cytosines, which further increases binding affinity for RNA and reduces the potential for immune stimulation (247). The oligonucleotide caused a reduction in target RNA, consistent with an RNase H mechanism of action and inhibited the production of hepatitis C viral proteins in hepatocyte cells transfected with a partial HCV genome containing the 5′-non-

coding region, core protein region, and the majority of the envelope region. HCV animal models were not readily available when this compound was being investigated preclinically. Therefore, a surrogate model was used to evaluate the potential in vivo efficacy of this oligonucleotide. A vaccinia virus model was used in which the HCV 5'-noncoding region, containing the IRES, and a portion of the core protein sequence was fused to firefly luciferase gene. Intraperitoneal injection of the recombinant vaccinia virus into mice, produced high levels of luciferase activity in livers. ISIS 14803 selectively inhibited luciferase expression in the livers isolated from infected mice (246). ISIS 14803 is currently in phase II trials, administered as an intravenous infusion, alone or in combination with interferon and ribavirin (248).

B. Use of Antisense Oligonucleotides for Cancer Therapy

An antisense oligonucleotide directed to p53 was one of the first antisense oligonucleotides to be administered systemically to patients. Preclinical studies with OL(1)p53, a 20-mer phosphorothioate oligodeoxynucleotide complementary to a portion in exon 10 of the p53 mRNA, inhibited proliferation of acute myelogenous leukemia cells in cell culture (136,249). Correspondingly, OL(1) p53 was found to reduce the level of p53 in leukemic cells, whereas a reverse sequence control failed to do so (136). A phase 1 study was conducted at the University of Nebraska Medical Center in which OL(1)p53 was infused at doses ranging from 0.05 mg/kg/h to 0.25 mg/kg/h for 10 days into patients with hematological malignancies. There were no apparent toxicities that could be directly attributed to the oligonucleotide. Two patients experienced a transient increase in hepatic transaminase concurrent with administration of the drug. In contrast to observations made with other phosphorothioate oligodeoxynucleotides, 17% to 59% of intact drug was detected in urine in this group of patients. There was an inverse correlation between plasma concentrations of oligonucleotide and cumulative leukemic growth of long-term marrow cultures. However, this correlation was not observed clinically as there were no morphological complete responses. These results provide evidence that OL(1)p53 was tolerated in leukemic patients; however, OL(1)p53 is no longer in active development.

Overexpression of *bcl*-2 is common in several cancers, in particular, non-Hodgkin lymphoma, and may contribute to decreased sensitivity to chemotherapeutic agents (250,251). An 18-mer phosphorothioate antisense oligodeoxynucleotide targeting the translation initiation codon of the *bcl*-2 gene was shown to inhibit the growth of lymphoma cells in severe combined immunodeficient (SCID) mice (252). Follow-up studies demonstrated that oblimersen inhibited growth of lymphoma cells in severely immunocompromised SCID and nonobese diabetic/SCID mice, suggesting that the activity of the oligonucleotide was not secondary to an immunostimulatory effect (253). The drug has also demonstrated antitumor activity in preclinical models of various other cancers such as melanoma, prostate cancer, and gastric cancer (252). Webb et al. conducted a phase 1 clinical trial of this oligonucleotide (Genta 3139, oblimersen) at the Royal Marsden Hospital in London. Genta 3139 was administered as a daily subcutaneous infusion for 14 days to patients with BCL-2 positive non-Hodgkin lymphoma. The dose of the drug given ranged from 4.6 mg/m^2 to 73.6 mg/m^2. Other than local inflammation at the site of infusion, no treatment-related side effects were noted. In 2 patients, tomography scans revealed reductions in tumor size with one complete response. In 2 additional patients, the number of circulating lymphoma cells decreased during treatment. Reduced levels of *bcl*-2 protein expression in circulating lymphoma cells were detected in 2 out of 5 patients. These findings again demonstrate that phosphorothioate oligodeoxynucleotides can be safely administered to patients and also provide preliminary efficacy data with a *bcl*-2 antisense oligonucleotide. Several other phase I/II studies on oblimersen have been performed, including studies in prostate cancer, breast cancer, colorectal cancer, AML, CML, multiple myeloma, and malignant melanoma (254,255). Side effects associated with the use of oblimersen included thrombocytopenia, hypotension, fever, and hypoglycemia (255). Promising clinical activity was seen in several of the studies, warranting continued investigation of the drug. The bcl-2 antisense oligonucleotide is currently in phase III trials for the treatment of melanoma, chronic lymphocytic leukemia, multiple myeloma, and non-small lung cancer, and in several additional phase II trials (255).

Protein kinase C (PKC) was originally identified as a serine/threonine kinase involved in mediating intracellular responses to a variety of growth factors, hormones, and neurotransmitters (256). Molecular cloning studies have revealed that PKC exists as a family of at least 11 closely related isozymes, which are subdivided on the basis of certain structural and biochemical similarities (257–260). Considerable experimental evidence exists for a role of PKC in some abnormal cellular process, such as inflammation, tumor promotion, and carcinogenesis (260–262). Antisense oligonucleotides have been identified that target individual members of the PKC family, both as research tools and as potential drugs (32,111,263,264). Protein kinase C-α has been implicated as a signaling molecule for a number of growth factors, and has been shown to regulate cell motility and apoptotic processes in human cells (265–271). To determine if inhibitors of protein kinase C-α could have potential value in the treatment of malignancies, we have identified an antisense oligonucleotide that specifically inhibit expression of PKC-α either in mouse or human cell lines (32,77,111). These antisense oligonucleotides have been used to identify cellular processes that are governed by this PKC isozyme (32,272–275).

The effects of the human-specific PKC-α phosphorothioate oligodeoxynucleotide, ISIS 3521/LY900003, has been examined on the growth of human tumor xenografts in nude mice. Analysis of PKC-α expression in the tumor tissue by immunohistochemistry revealed positive staining present in the cytoplasm and occasionally in the nuclei of tumor cells in animals treated with either saline or a scrambled control phosphorothioate oligodeoxynucleotide. In contrast, tumors treated with

ISIS 3521/LY900003 showed much reduced staining for PKC-α (276). In a second series of independent studies, ISIS 3521/LY900003 has been used to suppress the growth of U-87 glioblastoma tumor cells in nude mice (272). This cell line was chosen for study because it has previously been shown to be sensitive to growth inhibition by transfection with an antisense PKC-α cDNA. ISIS 3521/LY900003 reduced the growth of these tumor cells when implanted both subcutaneously and intracranially, whereas the scrambled control compound failed to inhibit tumor growth. This resulted in a doubling in median survival time of the animals with intracranially implanted tumors, with 40% long-term survivors of the treated animals. Levels of both ISIS 3521/LY90003 and the scrambled control oligodeoxynucleotide within tumor tissue were determined by capillary gel electrophoresis and found to both be about 2 μM after 21 daily intraperitoneal doses of 20-mg/kg oligodeoxynucleotide. ISIS 3521/LY900003 also reduced the expression of PKC-α in the tumor tissue, but not PKC-ε or PKC-ζ.

Based on the available biological evidence implicating PKC in the pathogenesis of certain tumor types and the broad spectrum of antitumor activity of ISIS 3521/LY90003 in the nude mouse xenograft implant model, clinical trials were initiated. A variety of tumors have been evaluated in phase I/II trials (277–280). In one trial, ISIS 3521/LY900003 was administered as a continuous 21-day infusion, then rested for 7 days. The cycle could be repeated if the treatments were tolerated and the tumor did not progress (277). The dose-limiting toxicities of ISIS 3521/LY90003 were thrombocytopenia and fatigue at a dose of 3.0 mg/kg/day. Pharmacokinetic measurements showed rapid plasma clearance and dose-dependent steady-state concentrations of ISIS 3521. Evidence of tumor response lasting up to 11 months was observed in 3 of 4 patients with ovarian cancer. There were no grade 3 or grade 4 toxicities reported. One patient displayed transient thrombocytopenia and 1 patient exhibited leukopenia. In a second phase I study, ISIS 3521/LY900003 was administered as a 2-h infusion 3 times per week for 3 consecutive weeks (278). A total of 36 patients received 99 cycles of the drug. Apparent drug-related toxicities included thrombocytopenia, nausea, vomiting, fever, chills, and fatigue. Dose escalation was stopped at a dose of 6 mg/kg because of concerns that peak plasma concentrations would approach those correlated with complement activation in monkeys (151). Most of the cancer patients had elevated baseline complement C3a. Following infusion of ISIS 3521/LY90003, several patients had a further increase in C3a; however, no clinical sequelae were attributed to the modest increases observed. Two non-Hodgkin lymphoma patients achieved complete responses, and 8 other patients showed stabilization of disease. Isis Pharmaceuticals, Inc., has completed several phase II trials including non-small cell lung carcinoma. Based on the phase II results, ISIS 3521/LY900003 is currently in 2 phase III trials for non-small cell lung carcinoma, one in combination with carboplatin and paclitaxel, and the second in combination with gemcitabine and taxol. ISIS 3521/LY9200003 is also being evaluated in additional phase II trials.

The discovery of viral oncogenes in the mid-1960s was a major breakthrough in understanding the molecular origins of cancer and directly led to the identification of the first human oncogene in 1982, ras (281). An antisense oligonucleotide targeting ha-ras gene product has initiated clinical trials. ISIS 2503 targets the AUG translation initiation codon for ha-ras gene product (282). Although the frequency of mutations in human cancers is significantly higher for the ki-ras gene product, we have found that antisense oligonucleotides targeting ha-ras gene exhibit broader antitumor effects when evaluated in human tumor xenograft models. In fact, the ha-ras antisense oligonucleotide was effective against human tumor xenografts known to contain a mutation in the ki-ras gene. A multicenter phase I trial against a broad spectrum of cancers has been completed (283). Patients received ISIS 2503 as a continuos intravenous infusion for 2 weeks, followed by a 1-week drug-free period. Patients will repeat the cycle as long as they tolerate the drug or when tumors fail to respond to therapies. In a second study, the drug was administered in a more convenient schedule (i.e., a weekly 24-hour infusion of ISIS 2503). Similar to the PKC-α and C-raf kinase antisense oligonucleotides, the drug was tolerated and exhibited enough, encouraging activity to warrant continuing phase II trials. Thus, a first-generation phosphorothioate oligodeoxynucleotide targeted to normal ha-*ras* is the first selective inhibitor of ras function to enter clinical trials.

Alterations in cellular cAMP concentrations have been associated with changes in cellular proliferation states. There are two isoforms of the major cAMP receptors, cAMP-dependent protein kinases I and II that are distinguished by different regulatory subunits (RI and RII). Increased expression of the RI subunit of PKA I correlates with cellular proliferation and cellular transformation, whereas a decrease in the RI subunit and an increase in the RII subunit correlates with growth inhibition and cellular differentiation (284). To directly address the role of the RI subunit in cell growth and differentiation, an antisense oligonucleotide targeting the RI subunit was designed. This oligonucleotide at concentration of 15 to 30 μM inhibited growth of several human cell lines without signs of cytotoxicity (285–287). As expected, the phosphorothioate oligodeoxynucleotide was more effective than the phosphodiester version. A single injection of the RI subunit phosphorothioate oligodeoxynucleotide suppressed growth of a human colon carcinoma xenograft for a week (286). Tumors exhibited normal growth rates when treated with a control oligonucleotide. Examining levels of PKA-I activity in the tumor xenografts provided further support of an antisense mechanism. The antisense oligonucleotide-treated tumors exhibited loss of enzyme activity 24 h after treatment. More recently, a second-generation, 2-*O*-methyl chimeric oligonucleotide (GEM231) targeting human PKA RI subunit has been described (288–290). This oligonucleotide was more effective than the first-generation oligonucleotide in suppressing growth of human tumor xenografts and has shown enhanced activity when combined with various chemotherapeutic agents. Clinical trials have been initiated with a 2′-*O*-methyl

chimeric PKA RI subunit antisense oligonucleotide (GEM 231) in the treatment of solid tumors.

C. Use of Antisense Oligonucleotides for Treatment of Inflammatory Diseases

In addition to targeting gene products implicated in viral replication or cancer, antisense oligonucleotides have been used to inhibit the expression of gene products, which may have utility for the treatment of inflammatory diseases. Intercellular adhesion molecule 1 (ICAM-1) is a member of the immunoglobulin gene family expressed at low levels on resting endothelial cells and can be markedly up-regulated in response to inflammatory mediators, such as TNF-α, interleukin 1, and interferon-γ on a variety of cell types. ICAM-1 plays a role in the extravasation of leukocytes from the vasculature to inflamed tissue and activation of leukocytes in the inflamed tissue (291–294). Alicaforsen (ISIS 2302) was identified out of a screen of multiple first-generation phosphorothioate oligodeoxynucleotides targeting various regions of the human ICAM-1 (2,295). Alicaforsen inhibits ICAM-1 expression by an RNase H-dependent mechanism of action (295). Alicaforsen will selectively inhibit ICAM-1 expression in a variety of cell types (295–297). Both sense and a variety of scrambled control oligonucleotides fail to inhibit ICAM-1 expression, including a 2-base mismatch control. Treatment of endothelial cells with alicaforsen blocked adhesion of leukocytes, demonstrating that blocking expression of ICAM-1 will attenuate adhesion of leukocytes to activated endothelial cells (295). ISIS 2302 also blocked a 1-way mixed lymphocyte reaction when the antigen-presenting cell was pretreated with ISIS 2302 to down-regulate ICAM-1 expression prior to exposure to the lymphocyte (Vickers et al., 1996 unpublished data). Thus, alicaforsen is capable of blocking both leukocyte adhesion to activated endothelial cells and costimulatory signals to T lymphocytes, both activities were predicted based on previous studies with monoclonal antibodies to ICAM-1.

To test the pharmacology of the human-specific antisense oligonucleotide, we have used experimental models in which immunocompromised mice contain human tissue xenografts. In one model, we were able to demonstrate a role for ICAM-1 in metastasis of human melanoma cells to the lung of mice (296). A second study addressed the role of ICAM-1 in production of cytotoxic dermatitis (lichen planus) in SCID mice containing human skin xenografts (298). Upon engraftment of the human tissue, heterologous lymphocytes injected into the graft migrate into the epidermis (epidermaltropism), and produced a cytotoxic interaction between effector lymphocytes and epidermal cells. Systemic administration of alicaforsen inhibited ICAM-1 expression in the human graft, decreased the migration of lymphocytes into the epidermis, and prevented subsequent lesion formation. A sense control oligodeoxynucleotide failed to attenuate the responses. These data demonstrate that an ICAM-1 antisense oligonucleotide administered systemically can attenuate an inflammatory response in the skin.

ISIS 3082 and ISIS 9125 are 20-base phosphorothioate oligodeoxynucleotides that hybridize to an analogous region in the 3′-untranslated region of murine and rat ICAM-1 mRNA, respectively (299,300). Similar to alicaforsen, ISIS 3082 and ISIS 9125 selectively inhibit ICAM-1 expression in mouse or rat cells by an RNase H-dependent mechanism. Rodent ICAM-1 antisense oligonucleotides have demonstrated activity in a mouse heterotopic heart transplant model (299), mouse pancreatic islet transplant model (301), and rat heart and kidney transplants (300). The murine ICAM-1 antisense oligonucleotide has also shown activity in mouse models of pneumonia, colitis, and arthritis (165,302). Haller et al. independently used an ICAM-1 antisense oligonucleotide to decrease acute renal injury following ischemia in rats (303).

Alicaforsen, which targets human ICAM-1, is currently being developed by Isis Pharmaceuticals, Inc., for the treatment of Crohn's disease and ulcerative colitis. Safety and pharmacokinetics of alicaforsen was established in a phase I study performed at Guy's hospital in normal volunteers (130). Volunteers were either infused over a 2-h period with escalating single doses or multiple doses given of alicaforsen or saline in a double-blinded trial. Brief dose-dependent increases in aPTT were seen at the time of peak plasma concentration and clinically insignificant increases in C3a were seen after repeated 2.0 mg/kg doses. C5a, blood pressure, and pulse were unaffected by administration of alicaforsen. No other adverse events or laboratory abnormalities related to the administration of the drug were noted. The Cmax was linearly related to dose and occurred at the end of infusion. Plasma half-life was approximately 53 min. Nonlinear changes in AUC and volume of distribution were noted with increasing dose, suggesting that oligonucleotide disposition might have a saturable component. These data suggest that ISIS 2302 was well tolerated in normal volunteers and that the pharmacokinetics in man was similar to that observed in nonhuman primates and rodents.

Alicaforsen was evaluated in a series of small phase IIa studies (20–40 patients in each trial) in rheumatoid arthritis, psoriasis, Crohn's disease, ulcerative colitis, and renal transplant. With the exception of the psoriasis study, the trials were placebo-controlled, double-blinded trials in which the drug is administered as a 2-h intravenous infusion. In all trials, the drug was well tolerated. In the rheumatoid arthritis trial, alicaforsen failed to produce significant efficacy but showed positive trends (304). The small sample size of the trial, 43 patients, did not allow definitive conclusions to be drawn.

In the phase IIa Crohn's disease study, conducted by Dr. Bruce Yacyshyn at the University of Edmonton, patients were administered 0.5 mg/kg, 1.0 mg/kg, and 2.0 mg/kg ISIS 2302 every other day for a total of 26 days (305). The response of the patients was not dose dependent, probably due to the narrow dose range investigated and the small number of patients in the lower dose groups (3 each). Therefore, all ISIS 2302-treated patients were analyzed as 1 group. Complete response, defined as Crohn's disease activity index (CDAI) score less than 150, was observed in 7 of 15 patients treated with ISIS 2302 and 0 of 4 of the placebo patients (305). At the end of

the study (6 months), 5 of the 7 patients were still in remission and 1 patient had a CDAI score of 156. During the treatment phase of the study, steroid doses were fixed, afterward the physician was allowed to adjust steroid dose based on symptoms. There was a statistically significant decrease in steroid use in patients treated with ISIS 2302 compared with placebo-treated patients at the end of the study. Other than expected increase in aPTT and mild facial flushing at the end of infusion in 1 patient, the drug was well tolerated. Based on these promising data, a large multicenter phase IIb trial of ISIS 2302 in Crohn's disease has been initiated. Thus, ICAM-1 antisense oligonucleotides may have therapeutic utility for the treatment of Crohn's disease.

The pilot trial in Crohn's disease was followed with a larger 299-patient, placebo-controlled, multicenter trial (306). Alicaforsen was administered intravenously 3 times per week at a dose of 2 mg/kg for either 2 weeks or 4 weeks. Patients were treated in months 1 and 3, with steroid withdrawal attempted by week 10. The primary endpoint of the trial was a CDAI less than 150 and off steroids at the end of week 14. Of the patients completing week 14, 64% of placebo patients had discontinued steroids and 78% of the alicaforsen-treated patients had successfully stopped steroid use ($p = 0.032$) (306). The number of patients achieving steroid-free remissions was similar in all 3 treatment arms. Pharmacodynamic analysis revealed that there was a strong correlation between drug exposure as measured by plasma AUC levels and response to alicaforsen treatment. Remissions increased from 13.0% (7/54) for the lowest AUC group to 55.6% (5/9) for the highest AUC group. One patient in the trial developed an IgM antibody to alicaforsen, without clinical sequelae. Adverse events reported in the trial were minimal with 2% of the patients exhibiting hypersensitive reactions. Anticipated increase in aPTT without bleeding episodes were noted and transient facial flushing during infusion were also noted. Although the trial was not positive, the correlation between exposure and clinical response warranted further investigation. As such, a 22-patient safety and pharmacokinetic trial was recently completed examining 250, 300, and 350 mg doses of alicaforsen. Doses were based on body weight. Infusion-related reactions of fever, chills, headache, nausea, emesis, and arthralgias were reported in 41% of the patients. Infusion-related reactions appear to be less frequent in patients receiving background steroids. The pharmacokinetic data suggest that patients receiving 300 to 350 mg of alicaforsen achieved adequate drug exposure, and this dose is currently under evaluation in a phase III trial.

Inhibitors of TNF-α have proven clinically useful for the treatment of rheumatoid arthritis, psoriasis, and Crohn's disease (307–311). Antisense oligonucleotides to TNF-α have demonstrated positive effects in mouse colitis models and a mouse model of stroke (312,313). ISIS 104803 is a second-generation chimeric, 2′-*O*-methoxyethyl/oligodeoxynucleotide targeting human TNF-α (116). A phase I study of ISIS 104803 has been completed in healthy males (116). The drug was dosed from 0.1 to 6.0 mg/kg given either intravenously of subcutaneously, with up to 4 doses given. Transient prolongation in aPTT was observed, similar to first-generation phosphorothioate oligodeoxynucleotides. Two patients experienced a rash, 1 a reversible platelet decrease, and tenderness was noted at the site of a subcutaneous injection. A decrease in TNF-α production was noted in peripheral blood leukocytes activated ex vivo with endotoxin in subjects treated with ISIS 104803. ISIS 104803 is currently under investigation in phase II trials for rheumatoid arthritis and psoriasis.

VIII. CONCLUSION

As is to be expected with first-generation technology, undesirable properties have been identified for phosphorothioate oligodeoxynucleotides (149,150,314,315). Despite these limitations, it is possible to use phosphorothioate oligodeoxynucleotides to selectively inhibit the expression of a targeted RNA in cell culture and in vivo. The pharmacokinetics of phosphorothioate oligodeoxynucleotides are similar across species and do not appear to exhibit major sequence-specific differences. When dosed at high levels, it is possible to identify toxicities in rodents and primates. However, at doses currently under evaluation in the clinic, phosphorothioate oligodeoxynucleotides have been well tolerated. In addition, there is evidence that phosphorothioate oligodeoxynucleotides provide clinical benefits to patients with viral infections, cancer, and inflammatory diseases. There are several phosphorothioate oligodeoxynucleotides in late-stage clinical trials, which will hopefully deliver more effective therapies for patients suffering from life-threatening or very debilitating diseases.

Extensive medicinal chemistry efforts have been successfully focused on identifying improved antisense oligonucleotides, which address some of these issues. There are at least 4 areas in which chemistry can add value to first-generation drugs: increase potency, decrease toxicity, alter pharmacokinetics, and lower costs. As an example, numerous modified oligonucleotides have been identified that have a higher affinity for target RNA than phosphorothioate oligodeoxynucleotides (84,87,91–93,316). Oligonucleotide modifications have been identified that exhibit increased resistance to serum and cellular nucleases, enabling use of oligonucleotides that do not have phosphorothioate linkages. The tissue distribution of oligonucleotides may be altered with either chemical modifications or formulations (79,134,140,141,143,144,181, 200,203). Preliminary data also suggest that oral delivery of antisense oligonucleotides may be feasible (141). Finally, a number of modified oligonucleotides have been described that potentially exhibited less toxicities than first-generation phosphorothioate oligodeoxynucleotides (78,149,178)]. However, as experience with these modified oligonucleotides is rather limited, it remains to be seen whether they will have a distinct toxicity profile.

In conclusion, first-generation phosphorothioate oligodeoxynucleotides have proven to be valuable pharmacological tools for the researcher and have produced new therapies for the patient. Identification of improved second-and third-generation oligonucleotides with novel formulation should better

therapies for patients. Although tremendous progress has been made for antisense technology during the past 14 years, there are many more questions that remain for the technology.

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